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Environmental Assessment of a Wood-Waste-Fired Industrial Watertube Boiler
Volume 2. Data Supplement

Acurex Corp., Mountain View, CA

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Research Triangle Park, NC

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ENVIRONMENTAL ASSESSMENT OF A WOOD-WASTE-FIRED INDUSTRIAL WATERTUBE BOILER

Volume II: Data Supplement

by

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16. ABSTRACT The two-volume report gives results from field tests of a wood-waste-fired industrial watertube boiler. Two series of tests were performed: one firing dry (11% moisture) wood waste, and the other firing green (34% moisture) wood waste. Emission measurements included: continuous monitoring of flue gas emissions; source assessment sampling system (SASS) sampling of the flue gas with subsequent laboratory analysis of samples to give total flue gas organics in two boiling point ranges, compound category information within these ranges, specific quantitation of the semivolatile organic priority pollutants, and flue gas concentrations of 73 trace elements; Method 5 sampling for particulate; controlled condensation system sampling for SO2 and SO3; and grab sampling of boiler mechanical collector hopper ash for inorganic and organic composition determinations. Flue gas CO emissions from the boiler were quite high, attributed to the high excess air levels at which the unit operated. NOx emissions were comparable with both fuels (175-200 ppm). SO2 and SO3 levels were less than 10 ppm, in keeping with the low sulfur content of sboth fuels. Total organic emissions decreased from 60-135 mg/dscm firing dry wood to 2-65 mg/dscm firing green wood, in parallel with corresponding boiler CO emissions.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS		b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group	
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Wood Wastes	Nitrogen Oxides	Stationary Sources	11L	
Water Tube Boilers		Particulate	13A	
Flue Gases	Trace Elements	Environmental Assessment	21B	06A
Assessments	Carbon Monoxide		14B	
Particles	Organic Compounds Polycyclic Compounds		14G	07C

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SECTION 1

INTRODUCTION

The purpose of this data supplement is to provide sufficient detail for researchers to perform their own analysis of the data obtained. Readers are referred to Volume I (Technical Results) for objectives, description of the source tested, results, interpretations, and conclusions.

This data supplement contains the following information:

Section 2: Preliminary Tests -- Stack velocity traverse and gas composition tests.

Section 3: Boiler Operating Data -- Field data sheets of boiler operating conditions from available test meters; boiler efficiency calculation using ASME abbreviated test forms.

Section 4: Sampling Data Sheets -- Emission data obtained with continuous monitoring instrumentation operated by EPA and GCA. Operating data tables for EPA Method 5 (for particulate mass emissions), Source Assessment Sampling Systems (SASS) (for particulate mass and size fractionation, trace elements, and organic emissions), and controlled condensation (for SO_2 and SO_3 sampling).

Section 5: Analytical Laboratory Results -- Fuel analyses; laboratory analysis reports on particulate emissions by gravimetric analysis; sulfur emissions by turbidimetric analysis; trace element emissions by spark source mass spectrometry (SSMS) and atomic absorption spectroscopy (AAS), and leachable anion analyses by specific ion electrode; C_1 to C_6 hydrocarbons by gas chromatography; total chromatographable organic (TCO) and gravimetric (GRAV) results; determination of organic compounds by gas chromatography/mass spectrometry (GC/MS) in total sample extracts; liquid chromatography (LC) separation; low resolution mass spectrometry (LRMS) of selected total extracts and LC fractions; radiological assay reports for flue gas particulate and flyash samples; biological assay reports for flue gas and solid flyash samples for both test 1 (dry wood) and test 2 (green wood).

SECTION 2

PRELIMINARY TESTS

TRAVERSE POINT LOCATION FOR CIRCULAR DUCTS

PLANT ETHAN ALLEN, OLD FORT, N.C.
DATE 4-13-81
SAMPLING LOCATION STACK
INSIDE OF FAR WALL TO
OUTSIDE OF NIPPLE, (DISTANCE A) 50.25"
INSIDE OF NEAR WALL TO
OUTSIDE OF NIPPLE, (DISTANCE B) 3¼"
STACK I.D., (DISTANCE A - DISTANCE B) 47"
NEAREST UPSTREAM DISTURBANCE
NEAREST DOWNSTREAM DISTURBANCE
CALCULATOR DAROS, BEST, CHIPS

SCHEMATIC OF SAMPLING LOCATION

TRAVERSE POINT NUMBER	FRACTION OF STACK I.D.	STACK I.D.	PRODUCT OF COLUMNS 2 AND 3 (TO NEAREST 1/8 INCH)	DISTANCE B	TRAVERSE POINT LOCATION FROM OUTSIDE OF NIPPLE (SUM OF COLUMNS 4 & 5)
1	From EPA	47"	.82 (1.0)	+3.25	4.25
	TABLES		1.50		4.75
			2.59		5.84
			3.71		6.96
			4.94		8.19
			6.20		9.45
			7.57		10.82
			9.12		12.37
			10.81		14.06
			12.78		16.03
			15.18		18.43
			18.71		21.96
			28.29		31.54
			31.82		35.07
			34.22		37.47
			36.19		39.44
			37.88		41.13
			39.43		42.68
			40.80		44.05
			42.07		45.32
			43.29		46.54
			44.42		47.67
			45.50		48.75
48		47"	46.48 (46.0	+3.25"	49.25

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PRELIMINARY VELOCITY TRAVERSE

PLANT ETHAN ALLEN, OLD FORT. NC.
DATE 4-13-81
LOCATION STACK - ROOF
STACK I.D. 47"
BAROMETRIC PRESSURE, in. Hg 28.60
STACK GAUGE PRESSURE, in. H_2O -.3" Hg
OPERATORS BEST / CHIPS (MAROS)

SCHEMATIC OF TRAVERSE POINT LAYOUT

TRAVERSE POINT NUMBER	VELOCITY HEAD (Δp_s), in. H_2O	STACK TEMPERATURE (T_s), °F
1	.55	320
	.55	322
	.60	323
	.70	323
	.60	323
	.65	323
	.65	323
	.55	323
	.45	323
	.45	323
	.40	322
	.30	322
	.30	322
	.15	320
	.10	318
	.10	318
	.05	318
	.05	317
	.05	315
	.05	315
	.03	314
	.03	305
	.02	N/R
48	.02	N/R
AVERAGE		

TRAVERSE POINT NUMBER	VELOCITY HEAD (Δp_s), in. H_2O	STACK TEMPERATURE (T_s), °F
1	.30	317
	.30	319
	.30	320
	.30	321
	.35	322
	.30	322
	.30	323
	.30	323
	.30	324
	.20	324
	.20	324
	.20	324
	.20	323
	.20	323
	.20	323
	.20	322
	.20	322
	.20	321
	.20	320
	.20	319
	.20	316
	.20	312
	.15	N/R
48	.10	N/R
AVERAGE		

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ISOKINECTIC SAMPLING WORKSHEET

Plant ETHAN ALLEN

Peformed by BEST.

Date 4-15-81

Sample Location

Test No./Type M-5

$$K = \frac{782.687\ (Cp)^2\ (1-B_{wo})^2\ P_s\ M_d}{K_o^2\ M_s\ P_m}$$

where: K = Contant of fixed and assumed parameters (dimensionless)

Pitot coefficient (dimensionless)	No 11 C_p	.79	assumed
Water vapor in the gas stream (proportion by volume)	B_{wo}	.90	assumed
Absolute stack gas pressure (in. Hg)	.3" H_2O P_s	28.90	
Molecular weight, stack gas dry (lb/lb-mole)	M_d	29.19	
Orifice coefficient (dimensionless)	K_o	.7126	(038)
Molecular weight, stack gas wet (lb/lb-mole) $M_d(1-B_{wo}) + 18(B_{wo})$	M_s	28.03	
Abolute meter pressure (in. Hg)	P_m	29.02	
$\frac{782.687\ (\_\_\_)^2\ (1-\_\_\_)^2\ (\_\_\_)\ (\_\_\_)}{(\_\_\_)^2\ (\_\_\_)\ (\_\_\_)}$	K	806.682	

$\alpha = .9928$

$CO_2 = 4.0\%$

$O_2 = 12.5\%$

$N_2 = 83.5\%$

ISOKINECTIC NOZZLE CALCULATION
AND
SAMPLING RATE CALCULATION

Plant Ethan Allen, Old Fort, N.C. Performed by Best

Date 4-15-81

Sample Location STACK

Test No./Type M-5

$$N_d = \left(\frac{\Delta H\ T_s}{K\ T_m\ \Delta P}\right)^{.25}$$

where: N_d = Nozzel diameter (inches)

Average pressure differential across the orifice meter (in. H_2O)	ΔH		
Temperature stack gas, average (^{o}F)	T_s		
Temperature of gas meter, average (^{o}F)	T_m		
Stack gas velocity pressure (in H_2O)	ΔP	.6	assumed
$\left(\frac{(\_\_\_)\ (\_\_\_ + 460)}{806.7\ (\_\_\_ + 460)(\_\_\_)}\right)^{.25}$	N_d		

$$\Delta H = K\ (N_d)^4\ \frac{T_m}{T_s}\ (\Delta P)$$

where: ΔH = Pressure differential across the orifice meter (in H_2O)

Nozzel diameter, actual (inches)	N_d	.3086
Temperature of gas meter (^{o}F)	T_m	
Temperature of stack gas (^{o}F)	T_s	
Stack gas velocity pressure (in H_2O)	ΔP	
$\left((\_\_\_)\ (\_\_\_)^4\ \frac{(\_\_\_ + 460)}{(\_\_\_ + 460)}\ (\_\_\_)\right)$	ΔH	
Magic number _______( )^4	$K(N_d)^4$	7.316

ISOKINECTIC SAMPLING WORKSHEET

Plant ETHAN ALLEN, OLD FORT, N.C. Peformed by R. BEST
Date 4-16-81
Sample Location STACK
Test No./Type 2/M-5

$$K = \frac{782.687\ (Cp)^2\ (1-B_{wo})^2\ P_s\ M_d}{K_o^2\ M_s\ P_m}$$

where: K = Contant of fixed and assumed parameters (dimensionless)

Pitot coefficient (dimensionless)	C_p	.79	
Water vapor in the gas stream (proportion by volume)	B_{wo}	.92	
$P_B = 29.60$ Absolute stack gas pressure (in. Hg)	$-.5"\ H_2O$ P_s	29.57	
Molecular weight, stack gas dry (lb/lb-mole)	M_d	30.0	assumed
Orifice coefficient (dimensionless)	K_o	.69	
Molecular weight, stack gas wet (lb/lb-mole) $M_d(1-B_{wo}) + 18(B_{wo})$	M_s	29.00	assumed
Abolute meter pressure (in. Hg)	P_m	29.70	
$\frac{782.687\ (\_\_\_)^2\ (1-\_\_\_)^2\ (\_\_\_)\ (\_\_\_)}{(\_\_\_)^2\ (\_\_\_)\ (\_\_\_)}$	K	894.41	

ISOKINECTIC NOZZLE CALCULATION
AND
SAMPLING RATE CALCULATION

Plant Ethan Allen, Old Fort, NC
Date 4-16-81
Sample Location STACK
Test No./Type 2/M-5

Performed by R. Best

$$N_d = \left(\frac{\Delta H \ T_s}{K \ T_m \ \Delta P}\right)^{.25}$$

where: N_d = Nozzel diameter (inches)

Average pressure differential across the orifice meter (in. H_2O)	ΔH	1.8
Temperature stack gas, average (^0F)	T_s	783
Temperature of gas meter, average (^0F)	T_m	555
Stack gas velocity pressure (in H_2O)	ΔP	.45
$\left(\frac{(\underline{\quad}) \ (\underline{\quad} + 460)}{(894.4) \ (\underline{\quad} + 460)(\underline{\quad})}\right)^{.25}$	N_d	.282

$$\Delta H = K \ (N_d)^4 \ \frac{T_m}{T_s} \ (\Delta P)$$

where: ΔH = Pressure differential across the orifice meter (in H_2O)

Nozzel diameter, actual (inches)	N_d	
Temperature of gas meter (^0F)	T_m	
Temperature of stack gas (^0F)	T_s	
Stack gas velocity pressure (in H_2O)	ΔP	
$\left((\underline{\quad}) \ (\underline{\quad})^4 \ \frac{(\underline{\quad} + 460)}{(\underline{\quad} + 460)} \ (\underline{\quad})\right)$	ΔH	
Magic number _______( )^4	$K(N_d)^4$	

SECTION 3

BOILER OPERATING DATA

BOILER OPERATING DATA : TEST (Preliminary)

Date: 4-13-81

Time Parameter	13:00	15:00	
Steam load (10^3 lb/hr)	11.0	14.0	
Underfire air (in. H_2O)	+ 0.6	+ 0.6	
Overfire air (in. H_2O)	+ 21	+ 21	
Press before coll. (in H_2O)	+ 2.0	+ 2.0	
Press after coll. (in H_2O)	+ 3.8	+ 3.5	
Furnace draft (in H_2O)	- 0.3 ± 0.1	- 0.25 ± 0.05	
Silo A feed (dry wood) (rpm/%)	500/30	500/30	
Silo B feed (wet wood) (rpm/%)	0	0	
Bridgewall temp (°F)	660	650	
Economizer inlet, H_2O (°F)	150	148	
Economizer outlet, H_2O (°F)	290	270	
Superheater steam outlet (°F)	500	515	
Superheater steam press (psig)	195	185	
Stack temp, aft. coll (°F)	450	435	
Comments:	stack traverse measurements of velocity and temperature		

BOILER OPERATING DATA : TEST No. 1 (Dry Wood)

Date : 4-15-81

Time / Parameter	9:40	10:10	12:30
Steam load (10^3 lb/hr)	17	~ 16	~ 15
Underfire air (in. H_2O)	+ 0.5	+ 0.6	+ 0.5
Overfire air (in. H_2O)	+ 22.5	+ 22.5	+ 22.0
Press before coll. (in H_2O)	+ 2.0	+ 2.0	+ 2.0
Press after coll. (in H_2O)	+ 3.5	+ 3.5	+ 3.5
Furnace draft (in H_2O)	- 0.25	- 0.25	- 0.25
Silo A feed (dry wood) (rpm/%)	480	700	600
Silo B feed (wet wood) (rpm/%)	0	0	0
Bridgewall temp (°F)	900	870	850
Economizer inlet, H_2O (°F)	155	155	155
Economizer outlet, H_2O (°F)	255	250	258
Superheater steam outlet (°F)	520	440	520
Superheater steam press (psig)	155	145	155
Stack temp, aft. coll (°F)	460	450	460
Comments :			NO = ~ 65 ppm O_2 = ~ 15 % CO = > 1000 ppm

BOILER OPERATING DATA : TEST No. 1 (Dry Wood)

Date : 4-15-81

Time Parameter	13:00	13:30	14:00
Steam load (10^3 lb/hr)	~ 15	~ 15	~ 14
Underfire air (in. H_2O)	+ 0.5	+ 0.5	+ 0.6
Overfire air (in. H_2O)	+ 22	+ 22.5	+ 22.5
Press before coll. (in H_2O)	+ 2.0	+ 2.0	+ 2.0
Press after coll. (in H_2O)	+ 3.6	+ 2.0 - 4.0	+ 3.5
Furnace draft (in H_2O)	- 0.1 - 0.4	- 0.1 - 0.35	- 0.25
Silo A feed (dry wood) (rpm/%)	520	520	440
Silo B feed (wet wood) (rpm/%)	0	0	0
Bridgewall temp (°F)	850	820	800
Economizer inlet, H_2O (°F)	155	150	150
Economizer outlet, H_2O (°F)	250	255	260
Superheater steam outlet (°F)	525	520	510
Superheater steam press (psig)	160	160	165
Stack temp, aft. coll (°F)	460	440	440
Comments :	Boiler blowdown test stopped at 1pm and resumed at 1:05 pm		

BOILER OPERATING DATA : TEST No. 1 (Dry Wood)

Date : 4-15-81

Time / Parameter	14:30	15:00	15:30
Steam load (10^3 lb/hr)	15	17	17
Underfire air (in. H_2O)	+ 0.6	+ 0.6	+ 0.5
Overfire air (in. H_2O)	+ 22	+ 22	+ 22
Press before coll. (in H_2O)	+ 2.0	+ 1.8	+ 2
Press after coll. (in H_2O)	+ 3.2	+ 3.0	+ 3.0
Furnace draft (in H_2O)	- 0.1 - 0.4	- 0.1 - 0.4	- 0.1 - 0.4
Silo A feed (dry wood) (rpm/%)	480	500	450
Silo B feed (wet wood) (rpm/%)	0	0	0
Bridgewall temp (°F)	800	800	800
Economizer inlet, H_2O (°F)	150	150	150
Economizer outlet, H_2O (°F)	260	265	265
Superheater steam outlet (°F)	510	500	510
Superheater steam press (psig)	165	165	165
Stack temp, aft. coll (°F)	435	430	430
Comments :			CM check O_2 = 13-17 CO = 500-2500

BOILER OPERATING DATA : TEST No. 1 (Dry Wood)

Date : 4-15-81

Time Parameter	16:00	16:30	17:00
Steam load (10^3 lb/hr)	17	15.5	17
Underfire air (in. H_2O)	+ 0.4-0.8	+ 0.3 - 0.8	+ 0.5
Overfire air (in. H_2O)	+ 22.5	+ 22	+ 22
Press before coll. (in H_2O)	+ 2.0	+ 2.0	+ 2.0
Press after coll. (in. H_2O)	+ 2.4	+ 3	+ 3
Furnace draft (in H_2O)	- 0 - 0.35	- 0- 0.4	- 0.25
Silo A feed (dry wood) (rpm/%)	500	390	500
Silo B feed (wet wood) (rpm/%)	0	0	0
Bridgewall temp. (°F)	800	780	780
Economizer inlet, H_2O (°F)	148	150	150
Economizer outlet, H_2O (°F)	260	260	265
Superheater steam outlet (°F)	510	520	495
Superheater steam press (psig)	162	170	165
Stack temp, aft. coll (°F)	430	430	430
Comments :			

BOILER OPERATING DATA : TEST No. 1 (Dry Wood)

Date: 4-15-81

Time Parameter	17:30	18:00	18:30
Steam load (10^3 lb/hr)	17	16.5	16
Underfire air (in. H_2O)	+ 0.6	+ 0.6	+ 0.6
Overfire air (in. H_2O)	+ 22.5	+ 22.5	+ 22.5
Press before coll. (in H_2O)	+ 1.8	+ 2.0	+ 2.0
Press after coll. (in H_2O)	+ 3	+ 3	+ 3
Furnace draft (in H_2O)	- 0.1 - 0.4	- 0.25	- 0.25
Silo A feed (dry wood) (rpm/%)	495	515	500
Silo B feed (wet wood) (rpm/%)	0	0	0
Bridgewall temp (°F)	770	760	750
Economizer inlet, H_2O (°F)	148	146	145
Economizer outlet, H_2O (°F)	270	265	265
Superheater steam outlet (°F)	500	485	490
Superheater steam press (psig)	165	162	162
Stack temp, aft. coll (°F)	430	425	420
Comments:			

BOILER OPERATING DATA : TEST No. 1 (Dry Wood)

Date : 4-15-81

Time Parameter	19:30	20:00	
Steam load (10^3 lb/hr)	16	17	
Underfire air (in. H_2O)	+ 0.6	+ 0.6	
Overfire air (in. H_2O)	+ 22.5	+ 22.5	
Press before coll. (in H_2O)	+ 2	+ 1.8 - 2.4	
Press after coll. (in H_2O)	+ 3	+ 3 - 4	
Furnace draft (in H_2O)	- 0.25	- 0.1 - 0.3	
Silo A feed (dry wood) (rpm/%)	510	520	
Silo B feed (wet wood) (rpm/%)	0	0	
Bridgewall temp. (°F)	750	740	
Economizer inlet, H_2O (°F)	145	145	
Economizer outlet, H_2O (°F)	265	265	
Superheater steam outlet (°F)	500	490	
Superheater steam press (psig)	165	156	
Stack temp, aft. coll (°F)	420	420	
Comments :	Wood fuel flowrate roughly calculated to be ~ 41 lb/BBdu or ~ 1700 lb/hr	End of test	

SUMMARY SHEET

ASME TEST FORM
FOR ABBREVIATED EFFICIENCY TEST

PTC 4.1-a (1964)

	TEST NO. 1 (dry wood)	BOILER NO. 1	DATE 4-15-81
OWNER OF PLANT ETHAN ALLEN	LOCATION OLD FORT, NC		
TEST CONDUCTED BY Alvarez / Castaldini, C.	OBJECTIVE OF TEST Emissions Meas.		DURATION 6 hrs
BOILER, MAKE & TYPE Wicks 55,000 lbs/hr coal stoker converted to wood fire	RATED CAPACITY 40,000 lb/hr		
STOKER, TYPE & SIZE FIXED GRATE WITH UNDERFIRE & OFA PORTS			
PULVERIZER, TYPE & SIZE NONE	BURNER, TYPE & SIZE SPREADER		
FUEL USED Dry wood chips	MINE	COUNTY	STATE / SIZE AS FIRED

PRESSURES & TEMPERATURES

1	STEAM PRESSURE IN BOILER DRUM	psia	NA
2	STEAM PRESSURE AT S. H. OUTLET	psia	130
3	STEAM PRESSURE AT R. H. INLET	psia	NA
4	STEAM PRESSURE AT R. H. OUTLET	psia	NA
5	STEAM TEMPERATURE AT S. H. OUTLET	F	503
6	STEAM TEMPERATURE AT R.H. INLET	F	NA
7	STEAM TEMPERATURE AT R.H. OUTLET	F	NA
8	WATER TEMP. ENTERING (ECON.) (BOILER)	F	152
9	STEAM QUALITY % MOISTURE OR P. P. M.		0
10	AIR TEMP. AROUND BOILER (AMBIENT)	F	
11	TEMP. AIR FOR COMBUSTION (This is Reference Temperature) †	F	60
12	TEMPERATURE OF FUEL	F	Amb.
13	GAS TEMP. LEAVING (Boiler) (Econ.) (Air Htr.)	F	436
14	GAS TEMP. ENTERING AH (If conditions to be corrected to guarantee)	F	NA

UNIT QUANTITIES

15	ENTHALPY OF SAT. LIQUID (TOTAL HEAT)	Btu/lb	
16	ENTHALPY OF (SATURATED) (SUPERHEATED) STM.	Btu/lb	1271
17	ENTHALPY OF SAT. FEED TO (BOILER) (ECON.)	Btu/lb	120
18	ENTHALPY OF REHEATED STEAM R.H. INLET	Btu/lb	—
19	ENTHALPY OF REHEATED STEAM R. H. OUTLET	Btu/lb	—
20	HEAT ABS/LB OF STEAM (ITEM 16 – ITEM 17)	Btu/lb	1151
21	HEAT ABS/LB R.H. STEAM (ITEM 19 – ITEM 18)	Btu/lb	—
22	DRY REFUSE (ASH PIT + FLY ASH) PER LB AS FIRED FUEL	lb/lb	
23	Btu PER LB IN REFUSE (WEIGHTED AVERAGE)	Btu/lb	
24	CARBON BURNED PER LB AS FIRED FUEL	lb/lb	
25	DRY GAS PER LB AS FIRED FUEL BURNED	lb/lb	2254

HOURLY QUANTITIES

26	ACTUAL WATER EVAPORATED	lb/hr	16,000
27	REHEAT STEAM FLOW	lb/hr	—
28	RATE OF FUEL FIRING (AS FIRED wt)	lb/hr	2267(2)
29	TOTAL HEAT INPUT $\frac{\text{Item 28} \times \text{Item 41}}{1000}$	kB/hr	17,500
30	HEAT OUTPUT IN BLOW-DOWN WATER	kB/hr	
31	TOTAL HEAT OUTPUT $\frac{(\text{Item 26} \times \text{Item 20}) + (\text{Item 27} \times \text{Item 21})}{1000}$ + Item 30	kB/hr	18,416

FLUE GAS ANAL. (BOILER) (ECON) (AIR HTR) OUTLET

32	CO_2	% VOL	3.8(3)
33	O_2	% VOL	16.4
34	CO	% VOL	0.24
35	N_2 (BY DIFFERENCE)	% VOL	79.6
36	EXCESS AIR	%	[illegible]

FUEL DATA

	wood ~~COAL~~ AS FIRED PROX. ANALYSIS	% wt		OIL	
37	MOISTURE	11.02	51	FLASH POINT F*	
38	VOL MATTER		52	Sp. Gravity Deg. API*	
39	FIXED CARBON		53	VISCOSITY AT SSU* BURNER SSF	
40	ASH	0.37	44	TOTAL HYDROGEN % wt	
	TOTAL		41	Btu per lb	
41	Btu per lb AS FIRED	7714(1)			
42	ASH SOFT TEMP.* ASTM METHOD			GAS	% VOL
	~~COAL OR OIL~~ AS FIRED (wood) ULTIMATE ANALYSIS		54	CO	
43	CARBON	45.27	55	CH_4 METHANE	
44	HYDROGEN	5.44	56	C_2H_2 ACETYLENE	
45	OXYGEN	37.73	57	C_2H_4 ETHYLENE	
46	NITROGEN	0.12	58	C_2H_6 ETHANE	
47	SULPHUR	0.036	59	H_2S	
40	ASH	0.33	60	CO_2	
37	MOISTURE	11.02	61	H_2 HYDROGEN	
	TOTAL			TOTAL	
	COAL PULVERIZATION			TOTAL HYDROGEN % wt	
48	GRINDABILITY INDEX*		62	DENSITY 68 F ATM. PRESS.	
49	FINENESS % THRU 50 M*		63	Btu PER CU FT	
50	FINENESS % THRU 200 M*		41	Btu PER LB	
64	INPUT-OUTPUT EFFICIENCY OF UNIT %	$\frac{\text{ITEM 31} \times 100}{\text{ITEM 29}}$ = 105.2			

	HEAT LOSS EFFICIENCY	Btu/lb A. F. FUEL	% of A. F. FUEL
65	HEAT LOSS DUE TO DRY GAS	3064	32.2
66	HEAT LOSS DUE TO MOISTURE IN FUEL	135	1.74
67	HEAT LOSS DUE TO H_2O FROM COMB. OF H_2	598	7.75
68	HEAT LOSS DUE TO COMBUST. IN REFUSE		0
69	HEAT LOSS DUE TO RADIATION		1.5
70	UNMEASURED LOSSES		1.5
71	TOTAL		44.7
72	EFFICIENCY = (100 – Item 71)		55.3

(1) 8,675 Btu/lb dry basis
(2) 2017 lb/hr dry basis
(3) Carbon balance
(4) Duct Calculation

* Not Required for Efficiency Testing

† For Point of Measurement See Par. 7.2.8.1-PTC 4.1-1964

PTC 4.1-b (1964)

CALCULATION SHEET

ASME TEST FORM
FOR ABBREVIATED EFFICIENCY TEST

Revised September, 1965

OWNER OF PLANT Ethan Allen TEST NO. 1 BOILER NO. DATE 4-15-81

30	HEAT OUTPUT IN BOILER BLOW-DOWN WATER = LB OF WATER BLOW-DOWN PER HR × $\left[\frac{\text{ITEM 15} \ldots - \text{ITEM 17} \ldots}{1000}\right]$ = ... kB/hr
24	*If impractical to weigh refuse, this item can be estimated as follows* DRY REFUSE PER LB OF AS FIRED FUEL = $\frac{\text{\% ASH IN AS FIRED COAL}}{100 - \text{\% COMB. IN REFUSE SAMPLE}}$ CARBON BURNED PER LB AS FIRED FUEL = $\frac{\text{ITEM 43} \; 45.27}{100} - \left[\frac{\text{ITEM 22} \; 5.4\times10^{-5} \times \text{ITEM 23} \; 8000}{14{,}500}\right]$ = 0.45 NOTE: IF FLUE DUST & ASH PIT REFUSE DIFFER MATERIALLY IN COMBUSTIBLE CONTENT, THEY SHOULD BE ESTIMATED SEPARATELY. SEE SECTION 7, COMPUTATIONS.
25	DRY GAS PER LB AS FIRED FUEL BURNED = $\frac{11CO_2 + 8O_2 + 7(N_2 + CO)}{3(CO_2 + CO)}$ × (LB CARBON BURNED PER LB AS FIRED FUEL + $\frac{3}{8}$ S) = $\frac{11 \times \text{ITEM 32} \; 3.5 + 8 \times \text{ITEM 33} \; 16.4 + 7\left(\text{ITEM 35} \; 79.6 + \text{ITEM 34} \; 0.2\right)}{3 \times \left(\text{ITEM 32} \; 3.8 + \text{ITEM 34} \; 0.2\right)} \times \left[\text{ITEM 24} \ldots + \frac{\text{ITEM 47} \ldots}{267}\right]$ = 27.5
36	EXCESS AIR† = $100 \times \frac{O_2 - \frac{CO}{2}}{.2682N_2 - (O_2 - \frac{CO}{2})} = 100 \times \frac{\text{ITEM 33} - \frac{\text{ITEM 34}}{2}}{.2682\,(\text{ITEM 35}) - (\text{ITEM 33} - \frac{\text{ITEM 34}}{2})}$ = 387

	HEAT LOSS EFFICIENCY	Btu/lb AS FIRED FUEL	$\frac{\text{LOSS}}{\text{HHV}}$ × 100 =	LOSS %
65	HEAT LOSS DUE TO DRY GAS = LB DRY GAS PER LB AS FIRED FUEL × C_p × $(t_{lvg} - t_{air})$ = ITEM 25 27.54 × 0.24 × Unit (ITEM 13) − (ITEM 11) 436 − 60 = ...	2475	$\frac{65}{41}$ × 100 =	32.2
66	HEAT LOSS DUE TO MOISTURE IN FUEL = LB H_2O PER LB AS FIRED FUEL × [(ENTHALPY OF VAPOR AT 1 PSIA & T GAS LVG) − (ENTHALPY OF LIQUID AT T AIR)] = $\frac{\text{ITEM 37}}{100}$ × [(ENTHALPY OF VAPOR AT 1 PSIA & T ITEM 13) − (ENTHALPY OF LIQUID AT T ITEM 11)] = ... 11.02/100(1250-28)	135	$\frac{66}{41}$ × 100 =	1.74
67	HEAT LOSS DUE TO H_2O FROM COMB. OF H_2 = $9H_2$ × [(ENTHALPY OF VAPOR AT 1 PSIA & T GAS LVG) − (ENTHALPY OF LIQUID AT T AIR)] = 9 × $\frac{\text{ITEM 44}}{100}$ × [(ENTHALPY OF VAPOR AT 1 PSIA & T ITEM 13) − (ENTHALPY OF LIQUID AT T ITEM 11)] = ... 9(5.44)/100 (1250 -28)	598	$\frac{67}{41}$ × 100 =	7.75
68	HEAT LOSS DUE TO COMBUSTIBLE IN REFUSE = ITEM 22 ...... × ITEM 23 ...... = ...	0.43	$\frac{68}{41}$ × 100 =	0
69	HEAT LOSS DUE TO RADIATION* = $\frac{\text{TOTAL BTU RADIATION LOSS PER HR}}{\text{LB AS FIRED FUEL} - \text{ITEM 28} \ldots}$	......	$\frac{69}{41}$ × 100 =	1.5
70	UNMEASURED LOSSES **	......	$\frac{70}{41}$ × 100 =	1.5
71	TOTAL	......	......	44.7
72	EFFICIENCY = (100 − ITEM 71)	......	......	55.3

BOILER OPERATING DATA : TEST 2 (Wet Wood)

Day : 4-16-81

Time / Parameter	12:30	13:00	13:30
Steam load (10^3 lb/hr)	11	10	10
Underfire air (in. H_2O)	+ 0.7	+ 0.7	+ 0.7
Overfire air (in. H_2O)	+ 22	+ 22.5	+ 22
Press before coll. (in H_2O)	+ 1.3	+ 1.8 ± 0.5	1.5
Press after coll. (in H_2O)	+ 2.5	+ 2.2	+ 2.2
Furnace draft (in H_2O)	- 0.10	- 0.15	- 0.15
Silo A feed (dry wood) (rpm/%)	0	0	0
Silo B feed (wet wood) (rpm/%)	550	500	550
Bridgewall temp. (°F)	1100	1050	1100
Economizer inlet, H_2O (°F)	155	150	150
Economizer outlet, H_2O (°F)	235	235	240
Superheater steam outlet (°F)	550	565	550
Superheater steam press (psig)	175	175	170
Stack temp, aft. coll (°F)	450	445	430
Comments :	Primary & OFA air valve settings were increased to 75 & 100% open from 50% open at Test 1. Resetted to 50% START OF TEST	Steam demand starting to drop again	

BOILER OPERATING DATA : TEST No. 2 (Wet Wood)

Date : 4-16-81

Time / Parameter	14:00	14:30	15:00
Steam load (10^3 lb/hr)	10 ± 2	7 ± 2	8 ± 3
Underfire air (in. H_2O)	+ 0.7	+ 0.6	+ 0.65
Overfire air (in. H_2O)	+ 22	+ 22.5	+ 22.5
Press before coll. (in H_2O)	+ 1.5	+ 1.8	+ 1.8
Press after coll. (in H_2O)	+ 2.2	+ 3.0	+ 3.0
Furnace draft (in H_2O)	- 0.15	- 0.15	- 0.20
Silo A feed (dry wood) (rpm/%)	0	0	0
Silo B feed (wet wood) (rpm/%)	550	620	540
Bridgewall temp (°F)	1100	1050	1020
Economizer inlet, H_2O (°F)	150	150	150
Economizer outlet, H_2O (°F)	240	235	245
Superheater steam outlet (°F)	560	560	550
Superheater steam press (psig)	170	165	175
Stack temp, aft. coll (°F)	430	435	440
Comments :	CM Check: CO ~ 1500 max ppm	Steam demand dropping rapidly	

BOILER OPERATING DATA : TEST No. 2 (Wet Wood)
Date : 4-16-81

Time / Parameter	15:30	16:30	17:00
Steam load (10^3 lb/hr)	8 ± 3	7 ± 3	7 ± 3
Underfire air (in. H_2O)	+ 0.6	0.6	0.5
Overfire air (in H_2O)	+ 22.5	+ 22.5	+ 22.5
Press before coll. (in H_2O)	+ 2.0	+ 1.8	+ 1.9
Press after coll. (in H_2O)	+ 3.0	+ 3	+ 3.0
Furnace draft (in H_2O)	- 0.25	- 0.2	- 0.2
Silo A feed (dry wood) (rpm/%)	0	0	0
Silo B feed (wet wood) (rpm/%)	540	550	550
Bridgewall temp. (°F)	1000	1000	1000
Economizer inlet, H_2O (°F)	150	155	152
Economizer outlet, H_2O (°F)	250	242	252
Superheater steam outlet (°F)	545	550	520
Superheater steam press (psig)	175	185	187
Stack temp, aft. coll (°F)	440	440	420
Comments :	At about 16:00 hr boiler fire went out Test stopped - Added dry chips for ~ 15 minutes	At 16:38 lost fire again. Went on dry chips for ~ 4 minutes Test stopped during that time	

BOILER OPERATING DATA : TEST No. 2 (Wet Wood)

Date 4-16-81

Time / Parameter	17:30	18:00	
Steam load (10^3 lb/hr)	8 ± 3.5	7.5 ± 3.5	
Underfire air (in. H_2O)	+ 0.5	+ 0.6	
Overfire air (in. H_2O)	+ 22.5	+ 22.5	
Press before coll. (in H_2O)	+ 1.9	+ 2.0	
Press after coll. (in H_2O)	+ 3 ± 0.3	+ 2.9	
Furnace draft (in H_2O)	- 0.2	- 0.18	
Silo A feed (dry wood) (rpm/%)	0	0	
Silo B feed (wet wood) (rpm/%)	600	500	
Bridgewall temp (°F)	1000	1000	
Economizer inlet, H_2O (°F)	150	155	
Economizer outlet, H_2O (°F)	257	255	
Superheater steam outlet (°F)	520	550	
Superheater steam press (psig)	185	190	
Stack temp, aft. coll (°F)	440	440	
Comments:		End of test	

SUMMARY SHEET

ASME TEST FORM
FOR ABBREVIATED EFFICIENCY TEST

PTC 4.1-a(1964)

TEST NO. 2 (Wet Wood) BOILER NO. 1 DATE 4-10-81
OWNER OF PLANT ETHAN ALLEN LOCATION OLD FORT, N.C.
TEST CONDUCTED BY Barrera / Castaldini, C. OBJECTIVE OF TEST [illegible] DURATION 6 hrs
BOILER, MAKE & TYPE Wicks 55000 lb/hr coal stoker converted to wood fire RATED CAPACITY ~45000 lb/hr
STOKER, TYPE & SIZE Fixed grate with underfire & OFA ports
PULVERIZER, TYPE & SIZE None BURNER, TYPE & SIZE [illegible]
FUEL USED MINE COUNTY STATE SIZE AS FIRED

PRESSURES & TEMPERATURES

1	STEAM PRESSURE IN BOILER DRUM	psia	NA
2	STEAM PRESSURE AT S. H. OUTLET	psia	198
3	STEAM PRESSURE AT R. H. INLET	psia	NA
4	STEAM PRESSURE AT R. H. OUTLET	psia	NA
5	STEAM TEMPERATURE AT S. H. OUTLET	F	550
6	STEAM TEMPERATURE AT R.H. INLET	F	NA
7	STEAM TEMPERATURE AT R.H. OUTLET	F	NA
8	WATER TEMP. ENTERING (ECON.)(BOILER)	F	152
9	STEAM QUALITY % MOISTURE OR P. P. M.		0
10	AIR TEMP. AROUND BOILER (AMBIENT)	F	
11	TEMP. AIR FOR COMBUSTION (This is Reference Temperature) †	F	80
12	TEMPERATURE OF FUEL	F	Amb
13	GAS TEMP. LEAVING (Boiler) (Econ.) (Air Htr.)	F	437
14	GAS TEMP. ENTERING AH (If conditions to be corrected to guarantee)	F	NA

UNIT QUANTITIES

15	ENTHALPY OF SAT. LIQUID (TOTAL HEAT)	Btu/lb	
16	ENTHALPY OF (SATURATED) (SUPERHEATED) STM.	Btu/lb	1297
17	ENTHALPY OF SAT. FEED TO (BOILER) (ECON.)	Btu/lb	120
18	ENTHALPY OF REHEATED STEAM R. H. INLET	Btu/lb	—
19	ENTHALPY OF REHEATED STEAM R. H. OUTLET	Btu/lb	—
20	HEAT ABS/LB OF STEAM (ITEM 16 – ITEM 17)	Btu/lb	1177
21	HEAT ABS/LB R. H. STEAM (ITEM 19 – ITEM 18)	Btu/lb	—
22	DRY REFUSE (ASH PIT + FLY ASH) PER LB AS FIRED FUEL	lb/lb	
23	Btu PER LB IN REFUSE (WEIGHTED AVERAGE)	Btu/lb	
24	CARBON BURNED PER LB AS FIRED FUEL	lb/lb	
25	DRY GAS PER LB AS FIRED FUEL BURNED	lb/lb	12.07

HOURLY QUANTITIES

26	ACTUAL WATER EVAPORATED	lb/hr	8500 (2)
27	REHEAT STEAM FLOW	lb/hr	NA
28	RATE OF FUEL FIRING (AS FIRED wt)	lb/hr	4314 (3)
29	TOTAL HEAT INPUT $\frac{(\text{Item 28} \times \text{Item 41})}{1000}$	kB/hr	24 732
30	HEAT OUTPUT IN BLOW-DOWN WATER	kB/hr	NA
31	TOTAL HEAT OUTPUT $\frac{(\text{Item 26} \times \text{Item 20}) + (\text{Item 27} \times \text{Item 21}) + \text{Item 30}}{1000}$	kB/hr	10004

FLUE GAS ANAL. (BOILER)(ECON) (AIR HTR) OUTLET

32	CO_2	% VOL	6.2 (4)
33	O_2	% VOL	13.9
34	CO	% VOL	0.15
35	N_2 (BY DIFFERENCE)	% VOL	79.2
36	EXCESS AIR	%	[illegible] (5)

FUEL DATA

	Wood ~~COAL~~ AS FIRED PROX. ANALYSIS	% wt		OIL	
37	MOISTURE	33.85	51	FLASH POINT F*	
38	VOL MATTER		52	Sp. Gravity Deg. API*	
39	FIXED CARBON		53	VISCOSITY AT SSU* BURNER SSF	
40	ASH		44	TOTAL HYDROGEN % wt	
	TOTAL		41	Btu per lb	
41	Btu per lb AS FIRED	5138 (1)			
42	ASH SOFT TEMP.* ASTM METHOD			GAS	% VOL
	~~COAL OR OIL~~ AS FIRED Wood ULTIMATE ANALYSIS		54	CO	
43	CARBON	35.07	55	CH_4 METHANE	
44	HYDROGEN	3.60	56	C_2H_2 ACETYLENE	
45	OXYGEN	26.06	57	C_2H_4 ETHYLENE	
46	NITROGEN	0.10	58	C_2H_6 ETHANE	
47	SULPHUR	0.02	59	H_2S	
40	ASH	1.24	60	CO_2	
37	MOISTURE	33.85	61	H_2 HYDROGEN	
	TOTAL			TOTAL	
	COAL PULVERIZATION			TOTAL HYDROGEN % wt	
48	GRINDABILITY INDEX*		62	DENSITY 68 F ATM. PRESS.	
49	FINENESS % THRU 50 M*		63	Btu PER CU FT	
50	FINENESS % THRU 200 M*		41	Btu PER LB	

64 INPUT-OUTPUT EFFICIENCY OF UNIT % $\frac{\text{ITEM 31} \times 100}{\text{ITEM 29}}$ = [illegible]

	HEAT LOSS EFFICIENCY	Btu/lb A. F. FUEL	% of A. F. FUEL
65	HEAT LOSS DUE TO DRY GAS	1173	[illegible]
66	HEAT LOSS DUE TO MOISTURE IN FUEL	407	7.9
67	HEAT LOSS DUE TO H_2O FROM COMB. OF H_2	589	6.79
68	HEAT LOSS DUE TO COMBUST. IN REFUSE	51	0.99
69	HEAT LOSS DUE TO RADIATION		2.0
70	UNMEASURED LOSSES		1.5
71	TOTAL		33.7
72	EFFICIENCY = (100 – Item 71)		66.3

(2) Average of meter reading over the duration of the test. Steady load increase from 6K lb/hr to 7K lb/hr during test

(3) lb/hr [illegible]

(1) 8,675 Btu/lb dry basis

* Not Required for Efficiency Testing

† For Point of Measurement See Par. 7.2.8.1-PTC 4.1-1964

(4) [illegible]

(5) [illegible]

PTC 4.1-b (1964)

CALCULATION SHEET

ASME TEST FORM
FOR ABBREVIATED EFFICIENCY TEST

Revised September, 1965

OWNER OF PLANT Ethan Allen   TEST NO. 2   BOILER NO.   DATE 4-16-81

30	HEAT OUTPUT IN BOILER BLOW-DOWN WATER = LB OF WATER BLOW-DOWN PER HR × $\left[\dfrac{\text{ITEM 15} - \text{ITEM 17}}{1000}\right]$ = ... kB/hr			
24	*If impractical to weigh refuse, this item can be estimated as follows* DRY REFUSE PER LB OF AS FIRED FUEL = $\dfrac{\text{\% ASH IN AS FIRED COAL}}{100 - \text{\% COMB. IN REFUSE SAMPLE}}$ CARBON BURNED PER LB AS FIRED FUEL = $\dfrac{\overset{\text{ITEM 43}}{35.05}}{100} - \left[\dfrac{\overset{\text{ITEM 22}}{0.0064} \times \overset{\text{ITEM 23}}{8000}}{14{,}500}\right] = .35$ NOTE: IF FLUE DUST & ASH PIT REFUSE DIFFER MATERIALLY IN COMBUSTIBLE CONTENT, THEY SHOULD BE ESTIMATED SEPARATELY. SEE SECTION 7, COMPUTATIONS.			
25	DRY GAS PER LB AS FIRED FUEL BURNED = $\dfrac{11CO_2 + 8O_2 + 7(N_2 + CO)}{3(CO_2 + CO)} \times (\text{LB CARBON BURNED PER LB AS FIRED FUEL} + \frac{3}{8}S)$ $= \dfrac{11 \times \overset{\text{ITEM 32}}{6.2} + 8 \times \overset{\text{ITEM 33}}{13.7} + 7\left(\overset{\text{ITEM 35}}{79.8} + \overset{\text{ITEM 34}}{0.1}\right)}{3 \times \left(\overset{\text{ITEM 32}}{6.2} + \overset{\text{ITEM 34}}{0.1}\right)} \times \left[\overset{\text{ITEM 24}}{0.35} + \dfrac{\overset{\text{ITEM 47}}{0.02}}{267}\right] = 13.19$			
36	EXCESS AIR † = $100 \times \dfrac{O_2 - \frac{CO}{2}}{.2682N_2 - (O_2 - \frac{CO}{2})} = 100 \times \dfrac{\text{ITEM 33} - \frac{\text{ITEM 34}}{2}}{.2682\,(\text{ITEM 35}) - (\text{ITEM 33} - \frac{\text{ITEM 34}}{2})} = 21.3$			
	HEAT LOSS EFFICIENCY	Btu/lb AS FIRED FUEL	$\frac{\text{LOSS}}{\text{HHV}} \times 100 =$	LOSS %
65	HEAT LOSS DUE TO DRY GAS = LB DRY GAS PER LB AS FIRED FUEL × C_p × $(t_{lvg} - t_{air})$ = $\overset{\text{ITEM 25}}{13.19} \times 0.24 \times \overset{\text{(ITEM 13)} - \text{(ITEM 11)}}{437 - 80}$ = ...	1173	$\frac{65}{41} \times 100 =$	20.4
66	HEAT LOSS DUE TO MOISTURE IN FUEL = LB H_2O PER LB AS FIRED FUEL × [(ENTHALPY OF VAPOR AT 1 PSIA & T GAS LVG) − (ENTHALPY OF LIQUID AT T AIR)] = $\frac{\text{ITEM 37}}{100}$ × [(ENTHALPY OF VAPOR AT 1 PSIA & T ITEM 13) − (ENTHALPY OF LIQUID AT T ITEM 11)] = ... 33.85/100 (1250 - 48)	407	$\frac{66}{41} \times 100 =$	7.09
67	HEAT LOSS DUE TO H_2O FROM COMB. OF H_2 = $9H_2$ × [(ENTHALPY OF VAPOR AT 1 PSIA & T GAS LVG) − (ENTHALPY OF LIQUID AT T AIR)] = 9 × $\frac{\text{ITEM 44}}{100}$ × [(ENTHALPY OF VAPOR AT 1 PSIA & T ITEM 13) − (ENTHALPY OF LIQUID AT T ITEM 11)] = ... 9(3.4/100 (1250-80))	389	$\frac{67}{41} \times 100 =$	6.79
68	HEAT LOSS DUE TO COMBUSTIBLE IN REFUSE = ITEM 22 ...... × ITEM 23 ...... = ...	51	$\frac{68}{41} \times 100 =$	0.89
69	HEAT LOSS DUE TO RADIATION* = $\dfrac{\text{TOTAL BTU RADIATION LOSS PER HR}}{\text{LB AS FIRED FUEL} - \text{ITEM 30}}$	114	$\frac{69}{41} \times 100 =$	2.0
70	UNMEASURED LOSSES **	[illegible]	$\frac{70}{41} \times 100 =$	1.5
71	TOTAL	......	......	38.7
72	EFFICIENCY = (100 − ITEM 71)	......	......	61.3

SECTION 4

SAMPLING DATA SHEETS

4.1 CONTINUOUS MONITORING EMISSION DATA (BY GCA AND EPA)

4.2 FIELD DATA SHEETS FOR EPA METHOD 5, SASS, AND CONTROLLED CONDENSATION

4.1 CONTINUOUS MONITORING EMISSION DATA (BY GCA AND EPA)

Emission results were compiled by GCA into summary tables.

TEST 1 (DRY WOOD)

FIFTEEN-MINUTE AVERAGE DATA FOR APRIL 15, 1981

Time	Elapsed time (min)	O_2 (MV)	NO_x (MV)	CO (MV)	O_2 (%)	NO_x (ppm)	CO (ppm)
1304	274	6.267	2.592	3.340	15.93	59.8	1366
1319	299	6.243	2.665	2.444	15.87	61.5	996
1334	314	6.258	2.443	3.431	15.91	56.3	1403
1349	329	6.273	2.207	4.520	15.95	50.7	1852
1401	341	6.589	1.899	5.707	16.75	43.5	2342
1413	353	6.281	2.211	4.073	15.97	50.8	1668
1425	365	6.442	2.196	4.681	16.38	50.5	1919
1440	380	6.440	2.237	4.785	16.37	51.4	1962
1455	395	6.613	1.595	6.828	16.81	36.3	2804
1510	410	6.481	1.926	6.036	16.48	44.1	2478
1525	425	6.400	2.043	5.188	16.27	46.9	2128
1540	440	6.519	1.925	5.712	16.57	44.1	2344
1555	455	6.334	2.042	5.387	16.10	46.8	2210
1610	470	6.444	1.777	6.407	16.38	40.6	2631
1625	485	6.232	2.237	4.773	15.85	51.4	1957
1640	500	6.488	2.463	5.103	16.50	56.7	2093
1655	515	6.682	1.548	7.593	16.99	35.2	3120
1710	530	6.397	1.962	6.581	16.26	45.0	2703
1725	545	6.688	1.433	7.195	17.00	32.5	2956
1740	560	6.406	2.072	7.085	16.29	47.5	2910
1755	575	6.382	2.101	6.336	16.23	48.2	2602
1816	590	6.555	1.734	7.288	16.70	39.6	2994
1825	605	6.564	1.720	7.778	16.72	39.3	3196
1840	620	6.476	1.888	7.053	16.50	43.2	2897
1855	635	6.563	1.777	7.684	16.72	40.5	3158
1910	650	6.464	2.196	6.764	16.31	50.5	2778
1925	665	6.315	2.048	6.759	16.09	47.0	2776
1940	680	6.682	1.103	7.580	17.02	24.7	3115
1955	695	6.861	0.846	8.375	17.48	18.7	3443

TEST 2 (WET WOOD)

FIFTEEN-MINUTE AVERAGE DATA FOR APRIL 16, 1981

Time	Elapsed time (min)	O_2 (MV)	NO_x (MV)	CO (MV)	O_2 (%)	NO_x (ppm)	CO (ppm)
1112	1502	5.392	3.693	1.806	13.53	85.8	731
1127	1517	4.956	3.891	1.116	12.43	90.4	447
1142	1532	4.774	5.596	0.811	12.00	130.6	322
1157	1547	4.671	4.245	0.716	11.71	98.8	283
1212	1562	5.111	3.884	1.108	12.82	90.3	444
1227	1577	4.742	3.983	0.991	11.89	92.6	396
1242	1592	5.231	3.759	2.909	13.13	87.2	1185
1257	1607	5.141	3.584	1.254	13.00	83.2	504
1312	1622	6.286	2.385	5.438	15.77	54.9	2227
1327	1637	6.313	2.420	5.512	15.84	55.7	2257
1342	1652	5.365	3.230	2.657	13.46	78.4	1082
1357	1667	5.141	3.747	1.065	12.90	87.0	426
1412	1682	5.244	3.677	1.201	13.16	85.4	482
1427	1697	5.794	2.998	2.913	14.54	69.4	1187
1442	1712	5.794	2.814	2.441	14.54	65.0	993
1457	1727	5.565	3.327	1.312	13.96	77.1	528
Calibrations performed 1500-1600 hrs							
1612	1802	5.549	3.534	1.555	13.92	82.0	628
1627	1817	5.749	2.906	2.120	14.42	67.2	861
1642	1832	6.220	1.630	4.255	15.61	37.1	1740
1657	1847	5.762	3.073	2.991	14.46	71.1	1219
1712	1862	5.970	3.275	2.597	14.98	75.9	1057
1727	1877	6.050	2.724	4.183	15.18	62.9	1710
1742	1892	6.011	2.861	4.479	15.08	66.1	1832
1757	1907	5.744	2.828	2.866	14.41	65.4	1168

4.2 FIELD DATA SHEETS FOR EPA METHOD 5, SASS, AND CONTROLLED CONDENSATION

$\Delta H = \frac{T_m}{T_s} \times M.N. \times \Delta P$ [
(7.36)

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ACUREX Corporation

PARTICULATE TEST FIELD DATA SHEET

813668

Plant: Ethan Allen
Date: 4-15-81
Test Location: Stack Outlet
Run Number: Method 6 #1
Stack Diameter inches: 48"
Duct Dimensions in x in: —
Start Time: 1735
Operator: Best

Barometric Pressure: 28.92
Static Pressure: -.30" H_2O
Stack Pressure:
Probe Number: 5' glass
Pitot Coefficient: 0.79
Pitot Number: 11
Meter Box Number: 038
Orifice Coefficient: .7126 γ = .9928

Nozzle Size & Number: P-90 .3086
Molecular Weight:
BWO:

FILTER DATA

NUMBER	TARE	FINAL WT.
MV-165-30		

IMPINGER VOLUMES		TIME	CO2
100	146		
100	106		
0	2		
SILICA GEL			
537.7	553.1		

[illegible]

SAMPLE POINT	CLOCK TIME	VELOCITY HEAD ΔP in. wg	ORIFICE METER ΔH in. wg	GAS METER VOLUME FT³	TEMPERATURES °F STACK	PROBE	IMPINGER	ORGANIC MODULE	OVEN	GAS METER IN	GAS METER OUT	PUMP VACUUM in. Hg	√ΔP	
1	0.0	.25	1.28	292.384	311	342			350	78	78	5	.500	initial leak
2	2.5	.27	1.37	294.0	312	322	-		340	75	78	5	.520	rate
3	5.0	.30	1.52	295.6	312	310			309	75	78	5	.548	.010 at 13" Hg
4	7.5	.30	1.52	297.3	311	299			285	74	78	5	.548	Pitot OK
5	10.0	.28	1.42	299.0	311 ~~279~~	279			351	74	78	5	.529	
6	12.5	.28	1.42	300.7	310	269			244	73	77	5	.529	
7	15.0	.24	1.22	302.4	309	251			261	73	77	5	.490	
8	17.5	.24	1.22	304.0	312	232			294	73	77	5	.490	
9	20.0	.20	1.01	305.6	312	258			303	73	77	4	.447	
10	22.5	.17	.86	307.1	311	284			286	73	77	4	.412	
11	25.0	.17	.86	308.4	310	273			269	72	76	4	.412	
12	27.5	.13	.66	309.7	309	239			249	73	76	3	.361	
13	30.0	.13	.66	310.9	309	221			232	73	76	3	.361	
14	32.5	.15	.76	312.1	309	263			242	73	76	3	.387	
15	35.0	.15	.76	313.3	309	254			245	72	76	3	.387	
16	37.5	.15	.76	314.5	308	226			253	72	75	3	.387	
17	40.0	.18	.91	315.8	308	246			273	72	79	4	.424	
18	42.5	.15	.76	317.1	308	268			268	72	75	3	.387	
AVG/TOTAL														

SAMPLE POINT	CLOCK TIME	VELOCITY HEAD ΔP in wg	ORIFICE METER ΔH in wg	GAS METER VOLUME FT³	TEMPERATURES °F							PUMP VACUUM in Hg	√ΔP	
					STACK	PROBE	IMPINGER	ORGANIC MODULE	OVEN	GAS METER				
										IN	OUT			
19	45.0	.17	.86	318.5	308	241			270	72	75	4	.412	
20	47.5	.15	.76	319.8	306	244			269	72	75	4	.387	
21	50.0	.15	.76	321.1	306	261			267	72	74	4	.387	
22	52.5	.10	.51	322.3	306	240			244	72	74	3	.316	
23	55.0	.10	.51	323.3	306	243			237	72	74	3	.316	
24	57.5	.10	.51	324.4	306	262			233	72	74	3	.316	
North [illegible]	60.0 [illegible]			326.403										
1	60.0	.38	1.93	326.403	308	257			268	74	75	6	.616	
2	62.5	.38	1.93	327.3	308	265			273	74	75	6	.616	
3	65.0	.45	2.28	329.2	309	271			273	72	74	8	.671	
4	67.5	.50	2.54	331.4	309	272			264	71	74	8	.707	
5	70.0	.50	2.54	333.5	308	270			249	71	74	8	.707	
6	72.5	.50	2.54	335.6	309	265			236	71	74	8	.707	
7	75.0	.50	2.54	337.8	306	259			227	71	74	8	.707	
8	77.5	.50	2.54	340.0	309	249			226	71	74	8	.707	
9	80.0	.45	2.28	342.2	311	250			241	71	74	8	.671	
10	82.5	.45	2.28	344.0	309	249			279	71	74	8	.671	
11	85.0	.35	1.78	346.4	310	249			301	71	74	7	.592	
12	87.5	.18	.91	348.4	307	249			273	71	74	4	.424	
13	90.0	.10	.51	349.8	306	248			277	71	74	3	.316	
14	92.5	.08	.41	350.9	309	256			262	70	73	3	.283	
15	95.0	.05	.25	351.8	307	249			243	69	73	3	.224	
16	97.5	.05	.25	352.6	304	248			232	68	72	3	.224	
17	100.0	.05	.25	353.3	306	247			220	68	72	3	.224	
18	102.5	.05	.25	354.1	307	245			222	67	71	3	.224	
19	105.0	.05	.25	354.8	305	245			238	67	71	3	.224	
20	107.5	.05	.25	355.5	302	245			253	67	71	3	.224	
AVG/TOTAL														

2/2

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ACUREX Corporation

PARTICULATE TEST FIELD DATA SHEET

Plant Ethan Allen

Date 4-15-81

Test Location Stack Outlet

Run Number Method 5 #1

Stack Diameter inches 48"

Duct Dimensions in x in

Start Time 1735

Operator Best

Barometric Pressure

Static Pressure

Stack Pressure

Probe Number

Pitot Coefficient 0.79

Pitot Number

Meter Box Number

Orifice Coefficient

Nozzle Size & Number

Molecular Weight

BWO

FILTER DATA

NUMBER	TARE	FINAL WT.

IMPINGER VOLUMES		TIME	CO2	O2	CO
SILICA GEL					

SAMPLE POINT	CLOCK TIME	VELOCITY HEAD ΔP in wg	ORIFICE METER ΔH in wg	GAS METER VOLUME FT³	TEMPERATURES °F							PUMP VACUUM in Hg	√ΔP	
					STACK	PROBE	IMPINGER	ORGANIC MODULE	OVEN	GAS METER				
										IN	OUT			
21	110.0	.05	.25	356.2	304	246			283	67	71	3	.224	
22	112.5	.05	.25	357.0	304	246			273	65	69	3	.224	
23	115.0	.05	.25	357.7	304	246			268	65	69	3	.224	
24	117.5	.05	.25	358.4	304	246			263	65	69	3	.224	
	120.0	End		359.209										
														Final leak
														rate
														.008 at
														12" Hg
										72.8				
AVG/TOTAL	120	0.215	1.09	66.825	308.0					71.2	74.4		.435	

813714 813713 813712

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ACUREX Corporation

PARTICULATE TEST FIELD DATA SHEET

Plant: Ethan allen
Date: 4-16-81
Test Location: Stack Outlet
Run Number: method 5 #2
Stack Diameter inches: 48"
Duct Dimensions in. x in.: ——
Start Time: 1535
Operator: Best

Barometric Pressure: 28.97
Static Pressure: -.30" H2O
Stack Pressure: 28.95
Probe Number: 5' glass
Pitot Coefficient:
Pitot Number: 11
Meter Box Number: 038
Orifice Coefficient: .7128 ∝ .9928

Nozzle Size & Number: R90 .3086
Molecular Weight:
BWO: 0.08

FILTER DATA

NUMBER	TARE	FINAL WT
MV-142-222		

IMPINGER VOLUMES		TIME	CO2	O2	CO
100	192				
100	111				
0	4				
		120.5			
SILICA GEL					
446.5	460.0 gr				

Traverse East Start on 1st point

SAMPLE POINT	CLOCK TIME	VELOCITY HEAD ΔP in. wg	ORIFICE METER ΔH in. wg	GAS METER VOLUME FT³	TEMPERATURES °F							PUMP VACUUM in. Hg	√ΔP	
					STACK	PROBE	IMPINGER	ORGANIC MODULE	OVEN	GAS METER IN	GAS METER OUT			
1	0.0	.10	.54	359.455	268	225			267	82	83	3	.316	initial leak
2	2.5	.10	.54	360.5	268	230			258	81	83	3	.316	rate
3	5.0	.10	.54	361.5	276	233			236	81	83	3	.316	.012 @ 12" Hg
4	7.5	.13	.68	362.6	298	236			226	80	82	3	.361	
5	10.0	.15	.78	363.8	304	235			237	80	82	3	.387	Pitot OK
6	12.5	.20	1.06	365.1	304	236			266	80	82	4	.447	
7	15.0	.20	1.06	366.5	295	237			284	81	82	4	.447	→ Burst of
8	17.5	.20	1.06	368.0	295	241			273	81	81	4	.447	heavy opacity
9	20.0	.20	1.06	369.5	297	244			255	81	81	4	.447	
10	22.5	.20	1.06	371.0	300	246			233	81	81	4	.447	
11	25.0	.15	.78	372.4	299	245			223	81	81	3	.387	
12	27.5	.15	.78	373.7	305	245			231	81	81	3	.387	
13	30.0	.13	.67	375.0	309	245			260	81	81	3	.361	
14	32.5	.15	.78	376.1	309	246			276	80	81	4	.387	
15	35.0	.17	.88	377.9	310	247			266	80	81	4	.412	
16	37.5	.20	1.05	37[illegible].6	311	249			247	80	81	4	.447	
17	40.0	.23	1.19	380.2	309	256			227	80	81	5	.480	
18	42.5	.25	1.29	381.7	311	264			220	80	81	5	.500	
AVG/TOTAL	42.5	0.167	0.877	22.265	298	240			250	81	81	4	0.405	

SAMPLE POINT	CK ME	VELOCITY HEAD ΔP in wg	ORIFICE METER ΔH in wg	GAS METER VOLUME FT³	TEMPERATURES °F STACK	PROBE	IMPINGER	ORGANIC MODULE	OVEN	GAS METER IN	GAS METER OUT	PUMP VACUUM in Hg	√ΔP	
19	45.0	.25	1.29	383.3	311	275			234	79	81	5	.500	
20	47.5	.25	1.29	385.0	309	275			262	79	81	5	.500	
21	50.0	.29	1.29	386.6	311	279			271	79	81	5	.500	
22	525	.28	1.45	388.2	310	281			261	77	81	6	.529	
23	55.0	.28	1.45	390.0	310	284			245	79	81	6	.529	
24	57.5	.28	1.45	391.6	310	287			227	78	80	6	.529	
	60.0 Stop			393.399										
Lower North 1	60.0	.04	.21	393.399	278	271			290	78	79	2	.200	
2	62.5	.04	.21	394.0	278	289			268	78	79	2	.200	
3	65.0	.04	.21	394.7	284	264			275	78	79	2	.200	
4	67.5	.04	.21	395.4	290	261			254	78	79	2	.200	
5	70.0	.04	.21	396.1	302	253			235	78	79	2	.20	
6	72.5	.04	.21	396.8	302	249			228	78	79	2	.20	
7	75.0	.04	.21	397.6	303	244			239	77	79	2	.20	
8	77.5	.04	.21	398.3	307	242			261	77	79	2	.20	
9	80.0	.04	.21	399.0	~~[illegible]~~ 307	~~[illegible]~~ 241			271	77	78	2	.20	
10	82.5	.64	.21	399.7	304	241			260	77	78	2	.20	
11	85.0	.06	.31	400.4	309	242			244	76	78	2	.245	
12	87.5	.06	.31	401.2	309	243			227	76	78	2	.245	
13	90.0	.13	.67	402.1	309	244			226	76	78	2	.361	
14	92.5	.20	1.04	403.2	316	244			246	76	77	5	.447	
15	75.0	.29	1.30	404.7	312	246			272	75	77	5	.500	
16	97.5	.30	1.56	406.4	312	250			284	75	77	6	.548	
17	100.0	.35	1.82	408.1	312	257			272	75	77	7	.592	
18	102.5	.40	2.01	410.0	312	266			257	75	77	7	.632	
19	105.0	.45	2.33	412.1	311	276			243	75	76	8	.671	
20	107.5	.50	2.59	414.2	311	267			240	75	76	9	.707	
		0.18	0.93	547.650	30[illegible]									
				THIS PAGE						77	78	5	0.386	
AVG/TOTAL	107.5													

age 2 of 2

ACUREX Corporation

PARTICULATE TEST FIELD DATA SHEET

Plant Ethan allen
Date 4-16-81
Test Location Stack Outlet
Run Number #2 m/5
Stack Diameter inches 48"
Duct Dimensions in x in
Start Time 1735
Operator Best

Barometric Pressure
Static Pressure
Stack Pressure
Probe Number
Pitot Coefficient
Pitot Number
Meter Box Number
Orifice Coefficient

Nozzle Size & Number
Molecular Weight
BWO

FILTER DATA

NUMBER	TARE	FINAL WT

IMPINGER VOLUMES		TIME	CO2	O2	CO
SILICA GEL					

SAMPLE POINT	CLOCK TIME	VELOCITY HEAD ΔP in wg	ORIFICE METER ΔH in wg	GAS METER VOLUME FT³	TEMPERATURES °F							PUMP VACUUM in Hg	√ΔP	
					STACK	PROBE	IMPINGER	ORGANIC MODULE	OVEN	GAS METER IN	GAS METER OUT			
21	110.0	.50	2.59	416.4	312	265			255	75	76	9	.707	
22	112.5	.50	2.59	418.6	310	269			273	75	76	9	.707	
23	115.0	.50	2.59	420.9	310	271			268	75	76	9	.707	
24	117.5	.50	2.59	423.1	310	275			263	75	76	9	.707	
	120.06 end			425.407										Final leak
														rate
														.007
														at 11" Hg
FINAL	TOTAL													
	120	0.282	1.466	65.972	304					77	77		0.420	
AVG/	120	0.50	2.59	65.972	310	272			265	75	76	9	707	

ACUREX Corporation

PARTICULATE TEST FIELD DATA SHEET

Plant	ETHAN ALLEN	Barometric Pressure	28.90
Date	APRIL 15, 1981	Static Pressure	-0.3"
Test Location	STACK - OUTLET	Stack Pressure	28.90
Run Number	1 - SASS	Probe Number	
Stack Diameter inches		Pitot Coefficient	
Duct Dimensions in. x in.		Pitot Number	P02
Start Time		Meter Box Number	067
Operator	J. Holm	Orifice Coefficient	1.007

Nozzle Size & Number P43 0.741

Molecular Weight 29.14

BWO

FILTER DATA

NUMBER	TARE	FINAL WT
mv 142-208		
mv 142-221		
mv 142-219		

	IMPINGER VOLUMES		TIME	CO₂	O₂	CO
1	500	630	+ 350 ml of H2O rinse			
2	500	645				
3	500	345				
	SILICA GEL					
	900	449.7				

W11

SAMPLE POINT	CLOCK TIME	VELOCITY HEAD ΔP in. wg	ORIFICE METER ΔH in. wg	GAS METER VOLUME FT³	TEMPERATURES °F							PUMP VACUUM in. Hg	√ΔP	
					STACK	PROBE	IMPINGER	ORGANIC MODULE	OVEN	GAS METER IN	GAS METER OUT			
0	1450			847.366										
15		0.20	1.3	897,385	315	397	--	62	395	92	89	10	.447	
30		0.23	1.3	947.76	314	395	--	53	398	98	90	12	.480	
45		0.21	1.3	998.0	311	395	--	52	398	99	90	12	.458	
60		0.19	1.3	047.16	306	395	--	52	397	95	88	17	.436	
78	16:10	0.20	1.3	100.4	312	393	--	55	390	81	79	13	.447	Δ FILTER
90	16.20 - 16.35	0.20	1.3	152	312	395	--	57	390	81	78	14	.447	
105	16.33 stop 15.15 start	0.18	1.3	206.46	313	395	--	57	390	85	80	14	.424	
120		0.18	1.3	262.05	313	395	--	56	390	90	80	16	.424	
135	15.45	0.22	1.3	317.65	316	402		53	390	87	79	17	.469	
150		0.20	1.3	368.58	310	401		50	388	88	80	19	.447	CONDENSATE DRAIN @
165		0.16	1.3	419.3	311	401		52	390	86	75	20	.400	1.80 STOP TEST FOR 3.3 MIN
180		0.20	1.3	468.276	310	401		56	392	80	75	13	.447	
195		0.20	1.3	520.64	315	400		57	396	81	74	14	.447	
210		0.15	1.3	572.43	310	400		60	399	77	70	15	.387	18.50-18.55
225		0.19	1.3	623.40	310	399		59	399	80	68	19	.436	NEW FILTER MV142-219
238	20.00	0.20	1.3	1667.004	310	400		59	399	80	68	20	.447	OC - SHUT DOWN AT
														2000 hrs
AVG/TOTAL			1.3	819.645	311.6					83			.44	

ACUREX Corporation

PARTICULATE TEST FIELD DATA SHEET

Plant Eman Allen
Date April 16, 1981
Test Location Stack - Outlet
Run Number 2 - SASS
Stack Diameter inches
Duct Dimensions in. x in.
Start Time 10:58
Operator J Horn

Barometric Pressure 28.975 @ 0900
Static Pressure -0.3"
Stack Pressure
Probe Number
Pitot Coefficient
Pitot Number
Meter Box Number 067
Orifice Coefficient

Nozzle Size & Number 0.741 Pucl(43)
Molecular Weight 29.17
BWO

FILTER DATA

NUMBER	TARE	FINAL WT
MT 143-20? MV 143-2??		
MV 143-307 MV 143-216		
MJ 143-315 MV 143-21A 143-217		

	IMPINGER VOLUMES		TIME	CO₂	O₂	CO
#1	500	1325				
#2	500	650				
#3	500	690				
		391				
	SILICA GEL					
	900	1125				

Imp. Rinse ≈160 ml
ml MeCl contaminated approx. 15 ml)

SAMPLE POINT	CLOCK TIME	VELOCITY HEAD ΔP in. wg	ORIFICE METER ΔH in. wg	GAS METER VOLUME FT³	TEMPERATURES °F							PUMP VACUUM in Hg	√ΔP	WEST PORT 3" NIPPLE 9½" FROM OUTSIDE NIPPLE TO
					STACK	PROBE	IMPINGER	ORGANIC MODULE	OVEN	GAS METER IN	GAS METER OUT			
	10:58			684.103										NOZZLE
15	12:33	0.20		740.55	308	400		60	400	82	81	14		Δ FILTER ON
30	12:47	0.20	1.85	797.156	311	400		65	400	85	81	20 14		@ 13:00
45	13:15	0.20	1.85	855.35	299	400		63	400	86	82			11 minutes into test stop Boiler put at
60	13:30	0.20	1.8	913.000	299	400		62	400	88	84	20 15		Δ FILTER full load 13.35
75	13:50	0.20	1.82	970.45	290	395		65	400	95	86	18		
90	14:09	0.22	1.7	1026.278	311	407		58	400	94	85	20 14		13 50 DRAIN
105	14:27	0.22	1.78	1084.56	313	400		62	400	91	86	18		CONDENSATE 14 09 Δ
120	14:43	0.25	1.6	1140.894	312	400		62	400	86	84	20 14		FILTER
135	15:07	0.26	1.75	1198.55	311	400		62	400	93	86	17		Δ FILTER
150	15:23	0.23	1.7	1255.952	310	400		60	400	89	85	20 14		14 43
165	15:45	0.22	1.82	1314.005	308	400		58	400	92	84	17		START UP
180	16:00	0.18	1.6	1370.472	310	400		59	400	90	86	20 14		AT 14:50
195	16:15	0.22	1.8	1429.0	311	400		60	400	92	83	18		
210	16:30	0.20	1.85	1485.374	311	400		60	400	86	83	20 14		1100 Power Out
225	1700	0.24	1.8	1543.23	315	400		60	400	86	80	18		11.05 Power on
240	17:15	0.25	1.55	1599.140	313	399		59	401	84	79	20 14		13 52 Power out
255	17:38	0.16	1.85	1657.08	309	400		59	401	84	79	18		On @ 13 57 12 27 Start
AVG/TOTAL														12 36 New Nozzle [illegible]

9½"

ACUREX Corporation

PARTICULATE TEST FIELD DATA SHEET

Plant
Date
Test Location
Run Number
Stack Diameter inches
Duct Dimensions in. x in.
Start Time
Operator

Barometric Pressure
Static Pressure
Stack Pressure
Probe Number
Pitot Coefficient
Pitot Number
Meter Box Number
Orifice Coefficient

Nozzle Size & Number
Molecular Weight
BWO

FILTER DATA

NUMBER	TARE	FINAL WT.

IMPINGER VOLUMES		TIME	CO₂	O₂	CO
SILICA GEL					

SAMPLE POINT	CLOCK TIME	VELOCITY HEAD ΔP in. wg	ORIFICE METER ΔH in. wg	GAS METER VOLUME FT³	TEMPERATURES °F							PUMP VACUUM in. Hg	√ΔP	B. Dabbs To operate at 4cfm @ meter
					STACK	PROBE	IMPINGER	ORGANIC MODULE	OVEN	GAS METER IN	GAS METER OUT			
270	17 53	0.15	1.55	1712.388	310	400		59	401	84	79	20		Δ filter MV 142-216
														Began test at 15 30
														Δ filter
														MV 142-215 Began Again
														@ 16:07
														Δ filter MV 142-212
														Condensate drained
														Restart 16:45
														Δ Filter MV 142-209
														17:23
														16:85 heavy
														Smoke Rone
														1637 Stop Smoking
AVG/TOTAL				1028.285	308					85.6			.458	

CONTROLLED CONDENSATION SYSTEM (CCS)
FIELD DATA SHEET

Plant Ethan Allen
Date 4-15-81
Sample Location Stack N-2
Run No. 1
Operator E.C. DuPont

Ambient Temperature 82°F @ 1220
Barometric Pressure 28.50
Meter Box Number 088
Meter Orifice Coefficient .708
Meter α Factor 1.002 12-25-80 cal.

Sampling Time, min	Clock Time (24-hr) clock	Gas Meter Reading (V_m), ft^3	Temperature (°F)							
					Filter		°C		Dry Gas Meter	
		Init.	Stack	Probe	Skin	Out	Recirc Water	Exit Coil	In	Out
0	1240	72.335								
5	1245	74.00	310	547	1047	538	60	103	94	91
10	1250	75.60		561	1120	531	60	105	94	91
15	1255	77.7		582	1230	520	60	105	93	91
① 25	1315	80.5		540	1640	632	60	105	95	92
30	1320	81.63		541	1447	636	60	105	95	92
40	1330	85.01		550	1461	666	60	105	95	93
60	1350	90.1		547	1495	682	60	105	98	95
65	1355	91.87	310	560	1498	689	60	105	98	95
Average 65 min		19.535	310				60°C	105	94	

① Stop for soot blow @ (1300 to 1305)

ISOKINETIC PERFORMANCE WORKSHEET & PARTICULATE CALCULATIONS

Plant Ethan Allen

Date 4-15-81

Sample Location

Test No./Type 1/ccs

Performed by DmRos

Barometric Pressure (in. Hg)	P_b	28.90
Meter volume (std), $17.64\left(\frac{V_m}{\alpha}\right)\left(\frac{P_b+\frac{\Delta H}{13.6}}{T_m+460}\right)$ $17.64\left(\frac{\overset{19.535}{(\_\_\_)}}{(1.007)}\right)\left(\frac{(28.90)+\frac{(.15)}{13.6}}{(94)+460}\right)$	$V_{m\ std}$	17.86
Volume of liquid collected (grams)	Vl_c	–
Volume of liquid at standard condition (scf) $Vl_c \times 0.04707$	$V_{w\ std}$	–
Stack gas proportion of water vapor $\frac{V_{w\ std}}{V_{w\ std}+V_{m\ std}}$, $\frac{(\_\_\_)}{(\_\_\_)+(\_\_\_)}$	B_{wo}	.0481 From 1/M-5 Test
Molecular weight, stack gas dry (lb/lb-mole) $(\%\ CO_2 \times 0.44) + (\%\ O_2 \times 0.32) + (\%\ N_2 + \%\ CO \times 0.28)$ $(4.0 \times 0.44) + (16.0 \times 0.32) + (80.0 + \text{—} \times 0.28)$	M_d	29.28
Molecular weight, stack gas wet (lb/lb-mole) $Md(1-B_{wo}) + 18(B_{wo})$, $(\_\_\_)(1-\_\_\_) + 18(\_\_\_)$	M_s	28.74
Absolute stack pressure (in. Hg) $P_b + \frac{P_{stack}\ (\text{in. } H_2O)}{13.6}$, $(\_\_\_) + \frac{(-.3)}{13.6}$	P_s	

7602/5/81/Rev 1

Temperature stack gas, average (^oF)	T_s	310
Stack velocity (fps) $85.49\,(C_p)\,(\sqrt{\Delta P_{s\ avg}})\ \sqrt{\frac{T_s avg + 460}{P_s\ \ M_s}}$ $85.49\,(\_\_\_)(\sqrt{\quad\quad})\ \sqrt{\frac{(\_\_\_) + 460}{(\_\_\_)(\_\_\_)}}$	$V_{s(avg)}$	Approximately Same as average of Method 5 Results
Total sample time (minutes)	θ	65
Nozzle diameter, actual (inches)	N_d	
Percent isokinetic (%) $\frac{17.33\,(T_s + 460)(V_w\ std + V_m\ std)}{\theta\quad V_s\quad P_s\quad N_d^2}$ $\frac{17.33\,(\_\_\_ + 460)((\_\_\_) + (\_\_\_))}{(\_\_\_)(\_\_\_)(\_\_\_)(\_\_\_^2)}$	%I	Not Applicable
Area of stack (ft^2) $\pi = 3.1416$ $\pi r^2 \div 144$, $\pi(\_\_\_)^2 \div 144$	A_s	
Stack gas volume at standard conditions (dscfm) $60\,(1 - B_{wo})Vs_{avg}\,A_s \left(\frac{528}{T_s\ avg + 460}\right)\left(\frac{Ps}{29.92}\right)$ $60\,(1 - \_\_\_)(\_\_\_)(\_\_\_)\left(\frac{528}{\_\_\_ + 460}\right)\left(\frac{(\_\_\_)}{(29.92)}\right)$	Q_s	
Particulate matter concentration, dry (gr/dscf) $15.432\ \frac{M_p(grams)}{Vm_{std}}$, $15.432\ \frac{(\_\_\_)}{(\_\_\_)}$	$C_{s(std)}$	
Emission rate of particulate matter (lb/hr) $0.00857\ (Q_s)\ C_{s(std)}$, $0.00857\ (\_\_\_)(\_\_\_)$	E_p	

CONTROLLED CONDENSATION SYSTEM (CCS)
FIELD CHECKPOINT SHEET

Checkpoint	Initials		Remarks
	Supervisor	QA Inspector	
LABORATORY PREPARATION			
• Inspect and clean CCC. Both filter holder and CCC are cleaned with hot chromic acid solution and D.I. H_2O.		✓	
• Rinse with acetone and air dry CCC.		✓	
• Place Tissuequartz filter in filter housing.		✓	
• Check seal between end of joint and filter.		✓	
• Do not use grease on joints.		✓	
• Inspect and clean all glass joints.		✓	
SITE SETUP			
• Rinse the inside of probe prior to run.		✓	
• Rinse probe with acetone until rinse solution is clear.		✓	
• Perform leak test.		✓	
• Leak rate must be less than 80 ml/min (0.003 cfm).		✓	
• Thermocouple leads attched to probe and filter.		✓	
• CCC water bath held at 60°C (140°F) ±1°C.		✓	
• Leak test train.		✓	
• Probe temperature maintained at 316°C (600°F) ±17°C.		✓	
• Gas temperature out of filter holder held at 228°C (550°F).		✓	
• Fresh solutions placed in impingers.		✓	
• Fresh absorbent replaced in final impinger.		✓	
• Adjust flowrate in system to 8 lpm.		✓	

DS 4-15-81
1200p

CONTROLLED CONDENSATION SYSTEM (CCS)
FIELD CHECKPOINT SHEET -- Continued

Checkpoint	Initials		Remarks
	Supervisor	QA Inspector	
SAMPLING RUN • Turn vacuum pump on just before inserting probe in stack.		✓	
• Check seal between probe and port to prevent any outside air from entering stack.		✓	
• Run test for 1 hour or until coils are frosted to 1/2 or 2/3 their length.		✓	
• After run, cap both ends of probe and lay in horizontal position.		✓	
• Rinse the CCC coils into the modified Erlenmeyer flask with a maximum of 40 ml D.I. H_2O.		✓	
• Was any of the solution lost (0 ml estimated)?		✓	
• After probe has cooled, it is rinsed with a maximum of 40 ml D.I. H_2O into a 25-ml Erlenmeyer flask. - Was any solution lost (0 ml estimated)?		✓	
- Clean support equipment priot to next run.		✓	
- Save filter for titration.		✓	

Comments:

CONTROLLED CONDENSATION SYSTEM (CCS)
FIELD DATA SHEET

Plant ETHAN ALLEN
Date 4-16-81
Sample Location STACK (N-2)
Run No. 2-CCC
Operator DuPos

Ambient Temperature ~80°F
Barometric Pressure 28.80"Hg @ 1410
Meter Box Number 088
Meter Orifice Coefficient .708
Meter α Factor 1.007 12-25-80 cal

Sampling Time, min \ Clock Time (24-hr) clock	Gas Meter Reading (V_m), ft^3	Temperature (°F)							
				Filter				Dry Gas Meter	
	Init.	Stack	Probe	Skin	Out	Recirc Water	Exit Coil	In	Out
0 \ 1245	99.19								
① → 7.0 \ 1252	101.20	308	450	955	387	60	101	87	87
START 1300 15.0 \ 1308	103.90	308	431	981	401	60	101	87	86
20.0 \ 1312	106.00	299	450	1155	420	60	104	87	86
30.0 \ 1322	109.10	299	445	1269	508	60	104	87	86
40.0 \ 1332	111.10	299	451	1322	520	60	105	87	86
50 \ 1342	113.70	299	501	1348	528	60	102	87	86
70 \ 1402	118.51	295	516	1339	518	60	104	88	87
Average 70 min	19.320	301				60°C		(87)	

① Stop for soot blow ~5 min

ISOKINETIC PERFORMANCE WORKSHEET & PARTICULATE CALCULATIONS

Plant ETHAN ALLEN　　Performed by DiaRos
Date 4-16-81
Sample Location STACK N-2
Test No./Type 2/CCS

Barometric Pressure (in. Hg)	P_b	28.80
Meter volume (std), $17.64\left(\frac{V_m}{\alpha}\right)\left(\frac{P_b+\frac{\Delta H}{13.6}}{T_m+460}\right)$ $17.64\left(\frac{(19.326)}{(1.007)}\right)\left(\frac{(28.80)+\frac{(.15)}{13.6}}{(87)+460}\right)$	$V_{m\ std}$	17.826
Volume of liquid collected (grams)	Vl_c	–
Volume of liquid at standard condition (scf) $Vl_c \times 0.04707$	$V_{w\ std}$	–
Stack gas proportion of water vapor $\frac{V_{w\ std}}{V_{w\ std}+V_{m\ std}}$, $\frac{(\_\_\_)}{(\_\_\_)+(\_\_\_)}$	B_{wo}	.08 assumed from M-5 Test 2
Molecular weight, stack gas dry (lb/lb-mole) $(\%\ CO_2 \times 0.44) + (\%\ O_2 \times 0.32) + (\%\ N_2 + \%\ CO \times 0.28)$ $(5.5 \times 0.44) + (15.0 \times 0.32) + (BAL + \_\_\_ \times 0.28)$	M_d	29.48
Molecular weight, stack gas wet (lb/lb-mole) $Md(1-B_{wo}) + 18(B_{wo})$, $(\_\_\_)(1-\_\_\_) + 18(.08)$	M_s	28.56
Absolute stack pressure (in. Hg) $P_b + \frac{P_{stack}\ (\text{in. } H_2O)}{13.6}$, $(\_\_\_) + \frac{(-0.3)}{13.6}$	P_s	

7602/5/81/Rev 1

Temperature stack gas, average (^{o}F)	T_s	308
Stack velocity (fps) $85.49\ (C_p)\ (\sqrt{\Delta P}_{s\ avg})\ \sqrt{\frac{T_s avg + 460}{P_s\quad M_s}}$ $85.49\ (\_\_\_)(\sqrt{\quad})\ \sqrt{\frac{(\_\_\_) + 460}{(\_\_\_)(\_\_\_)}}$	$V_{s(avg)}$	
Total sample time (minutes)	θ	70
Nozzle diameter, actual (inches)	N_d	
Percent isokinetic (%) $\frac{17.33\ (T_s + 460)(V_w\ std + V_m\ std)}{\theta\quad V_s\quad P_s\quad N_d^2}$ $\frac{17.33\ (\_\_\_ + 460)((\_\_\_) + (\_\_\_))}{(\_\_\_)(\_\_\_)(\_\_\_)(^2\_\_\_)}$	%I	
Area of stack (ft^2) $\pi = 3.1416$ $\pi r^2 \div 144$, $\pi(\_\_\_)^2 \div 144$	A_s	
Stack gas volume at standard conditions (dscfm) $60\ (1 - B_{wo}) Vs_{avg}\ A_s \left(\frac{528}{T_s\ avg + 460}\right)\left(\frac{Ps}{29.92}\right)$ $60\ (1 - \_\_\_)(\_\_\_)(\_\_\_)\left(\frac{528}{\_\_\_ + 460}\right)\left(\frac{(\_\_\_)}{(29.92)}\right)$	Q_s	
Particulate matter concentration, dry (gr/dscf) $15.432\ \frac{M_p(grams)}{Vm_{std}}$, $15.432\ \frac{(\_\_\_)}{(\_\_\_)}$	$C_{s(std)}$	
Emission rate of particulate matter (lb/hr) $0.00857\ (Q_s)\ C_{s(std)}$, $0.00857\ (\_\_\_)(\_\_\_)$	E_p	

CCC - 2

CONTROLLED CONDENSATION SYSTEM (CCS)
FIELD CHECKPOINT SHEET

Checkpoint	Initials: Supervisor	Initials: QA Inspector	Remarks
LABORATORY PREPARATION			
• Inspect and clean CCC. Both filter holder and CCC are cleaned with hot chromic acid solution and D.I. H_2O.		✓	
• Rinse with acetone and air dry CCC.		✓	
• Place Tissuequartz filter in filter housing.		✓	
• Check seal between end of joint and filter.		✓	
• Do not use grease on joints.		✓	
• Inspect and clean all glass joints.		✓	
SITE SETUP			
• Rinse the inside of probe prior to run.		✓	
• Rinse probe with acetone until rinse solution is clear.		✓	
• Perform leak test.		✓	
• Leak rate must be less than 80 ml/min (0.003 cfm).	✓	.002 cfm	@ 15" Hg
• Thermocouple leads attched to probe and filter.		✓	
• CCC water bath held at 60°C (140°F) ±1°C.		✓	
• Leak test train.		✓	
• Probe temperature maintained at 316°C (600°F) ±17°C.		✓	
• Gas temperature out of filter holder held at 228°C (550°F).		✓	
• Fresh solutions placed in impingers.		✓	
• Fresh absorbent replaced in final impinger.		✓	
• Adjust flowrate in system to 8 lpm.		✓	

CCC-2

CONTROLLED CONDENSATION SYSTEM (CCS)
FIELD CHECKPOINT SHEET -- Continued

Checkpoint	Initials		Remarks
	Supervisor	QA Inspector	
SAMPLING RUN • Turn vacuum pump on just before inserting probe in stack.		✓	
• Check seal between probe and port to prevent any outside air from entering stack.		✓	
• Run test for 1 hour or until coils are frosted to 1/2 or 2/3 their length.		✓	
• After run, cap both ends of probe and lay in horizontal position.		✓	
• Rinse the CCC coils into the modified Erlenmeyer flask with a maximum of 40 ml D.I. H_2O.		✓	
• Was any of the solution lost (0 ml estimated)?		✓	
• After probe has cooled, it is rinsed with a maximum of 40 ml D.I. H_2O into a 25-ml Erlenmeyer flask.		✓	
- Was any solution lost (0 ml estimated)?		✓	
- Clean support equipment priot to next run. - Save filter for titration.		✓	

Comments:

SECTION 5

ANALYTICAL LABORATORY RESULTS

5.1 FUEL ANALYSIS

5.2 PARTICULATE EMISSIONS FROM SASS SAMPLES

5.3 PARTICULATE EMISSIONS FROM EPA METHOD 5 SAMPLES

5.4 SULFUR OXIDE EMISSIONS FROM CONTROLLED CONDENSATION SAMPLES

5.5 TRACE ELEMENT AND LEACHABLE ANION ANALYSES

5.6 GASEOUS (C_1 to C_6) HYDROCARBONS

5.7 TOTAL CHROMATOGRAPHABLE (TCO) AND GRAVIMETRIC ORGANICS, INFRARED SPECTRA (IR), AND GAS CHROMATOGRAPHY/MASS SPECTROMETRY (GC/MS) OF TOTAL SAMPLE EXTRACTS

5.8 LIQUID CHROMATOGRAPHY (LC) SEPARATION AND INFRARED SPECTRA OF LC FRACTIONS

5.9 LOW RESOLUTION MASS SPECTROMETRY (LRMS) OF SELECTED TOTAL SAMPLE EXTRACTS AND LC FRACTIONS

5.10 RADIOMETRIC ANALYSIS RESULTS

5.11 BIOLOGICAL ASSAY RESULTS

5.1 FUEL ANALYSIS

LABORATORY CERTIFICATE

CURTIS & TOMPKINS, LTD.
ESTABLISHED 1878
ANALYTICAL - **CHEMISTS** - CONSULTING
SAMPLERS — INSPECTORS

MEMBERS OFFICIAL CHEMISTS
AND/OR SAMPLERS FOR MANY
COMMODITY AND TRADE ORGANIZATIONS
CORPORATE OR STAFF MEMBERSHIP IN
PRINCIPAL SCIENTIFIC SOCIETIES

290 DIVISION STREET
SAN FRANCISCO, CALIF. 94103
U.S.A.
Telephone (415) 861-1863

CABLE ADDRESS ANALYST
REFEREE ANALYSES
RESEARCH — INVESTIGATIONS
VITAMIN ASSAYS — BIOCHEMISTRY
SPECIALISTS IN BULK COMMODITIES

Laboratory No. 81h100
Preliminary No. 6487

Reported 8/13/81
Sampled ------
Received 7/07/81

For ACUREX CORPORATION

Report on 5 samples of Fuel Product

Mark Project No. 7734.12, 7/06/81, Blanket Subcontract RB59186A, Release No. 2.

DRY BASIS EXCEPT AS NOTED

	Dry Wood - Test 1 813661			Wet Wood - Test 2 813743		
	1st Test	2nd Test	3rd Test	1st Test	2nd Test	3rd Test
Carbon (C), %	50.88	----	----	53.02	----	----
Hydrogen (H), %	6.11	----	----	5.44	----	----
Oxygen (O), (by difference), %	42.46	----	----	39.40	----	----
Nitrogen (N), %	0.14	0.08	0.16	0.16	0.09	0.20
Sulfur (S), %	0.04	0.04	0.03	0.03	0.02	0.03
Heating Value: BTU/Pound	8,675	----	----	8,675	----	----
Bulk density lbs/cu ft. (as rec'd)	14.52	----	----	11.95	----	----
Ash, %	0.37	----	----	1.95	----	----
Moisture (as rec'd)	11.02	----	----	33.85	----	----

SAMPLES DISCARDED 30 DAYS AFTER RECEIPT UNLESS OTHERWISE REQUESTED

Curtis & Tompkins Ltd.

5.2 PARTICULATE EMISSIONS FROM SASS SAMPLES

ACUREX Corporation
ANALYSIS LABORATORIES

DATA REPORTING FORM

CUSTOMER CMEA
DATE July 13, 1981
CUSTOMER CONTRACT NO. 307736.12
ACUREX CONTRACT NO. A81-05-030
RESULTS REPORT TO L. Waterland
TELEPHONE
ADDRESS
Ethan Allen - 1 SASS

SAMPLE ID (CUSTOMER)	Probe	1u	3u	10u	Filter	XAD				
SAMPLE ID (LAB)	648	644	645	646	660	650				
PARAMETER										UNITS
Weight	0.2308	0.4975	0.8018	0.7865	1.1061	130				gram

ANALYST J. Labash
REVIEWER G. Nicoll

Form EED-037 4/80

ISOKINETIC PERFORMANCE WORKSHEET & PARTICULATE CALCULATIONS

Plant Ethan Allen

Performed by DaRos

Date 4-15-81

Sample Location Stack

Test No./Type 1-SASS

Barometric Pressure (in. Hg)	P_b	28.90
Meter volume (std), $17.64 \left(\frac{V_m}{\alpha}\right)\left(\frac{P_b + \frac{\Delta H}{13.6}}{T_m + 460}\right)$ $17.64 \left(\frac{(819.64)}{(1.007)}\right)\left(\frac{(28.90) + \frac{(-.3)}{13.6}}{(83.) + 460}\right)$	V_m std	763.592
Volume of liquid collected (grams)	Vl_c	669.7
Volume of liquid at standard condition (scf) $Vl_c \times 0.04707$	V_w std	31.52
Stack gas proportion of water vapor $\frac{V_{w\ std}}{V_{w\ std} + V_{m\ std}} = \frac{(31.52)}{(31.52) + (763.592)}$	B_{wo}	0.04 use 4.81 %
Molecular weight, stack gas dry (lb/lb-mole) $(\%\ CO_2 \times 0.44) + (\%\ O_2 \times 0.32) + (\%\ N_2 + \%\ CO \times 0.28)$ $(4.5 \times 0.44) + (16.0 \times 0.32) + (79.5 + \text{—} \times 0.28)$	M_d	29.36
Molecular weight, stack gas wet (lb/lb-mole) $Md(1-B_{wo}) + 18(B_{wo}) = (29.36)(1-.0481) + 18(.0481)$	M_s	28.81
Absolute stack pressure (in. Hg) $P_b + \frac{P_{stack}\ (\text{in. } H_2O)}{13.6} = (28.90) + \frac{(-.3)}{13.6}$	P_s	28.88

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Temperature stack gas, average (°F)	T_s	311.6
Stack velocity (fps) $85.49\ (C_p)\ (\sqrt{\Delta P}_{s\ avg})\ \sqrt{\frac{T_s avg + 460}{P_s\ \ M_s}}$ $85.49\ (0.79)(0.44)\ \sqrt{\frac{(311.6) + 460}{(28.86)(28.81)}}$	$V_{s(avg)}$	28.63
Total sample time (minutes)	θ	238
Nozzle diameter, actual (inches)	N_d	0.741
Percent isokinetic (%) $\frac{17.33\ (T_s + 460)(V_w \text{ std} + V_m \text{ std})}{\theta\quad V_s\quad P_s\quad N_d^2}$ $\frac{17.33\ (311.6 + 460)((31.52) + (263.59))}{(238)(28.63)(28.86)(^2.741)}$	%I	98.5
Area of stack (ft²) $\pi = 3.1416$ $\pi r^2 \div 144,\quad \pi(\_\_\_\_)^2 \div 144$	A_s	12.57
Stack gas volume at standard conditions (dscfm) $60\ (1 - B_{wo}) Vs_{avg}\ A_s \left(\frac{528}{T_s \text{ avg} + 460}\right) \left(\frac{Ps}{29.92}\right)$ $60\ (1 - .0781)(28.63)(12.57)\left(\frac{528}{311.6 + 460}\right) \left(\frac{(28.86)}{(29.92)}\right)$	Q_s	13,567
Particulate matter concentration, dry (gr/dscf) $15.432\ \frac{M_p (\text{grams})}{Vm_{std}},\quad 15.432\ \frac{(5.4227)}{(263.592)}$	$C_{s(std)}$	0.0692.
Emission rate of particulate matter (lb/hr) $0.00857\ (Q_s)\ C_{s(std)},\ 0.00857\ (13,567)(0.0692)$	E_p	8.046

7602/5/81/Rev 1

ACUREX Corporation
ANALYSIS LABORATORIES

DATA REPORTING FORM

CUSTOMER CMEA
DATE July 13, 1981
CUSTOMER CONTRACT NO. 307736.12
ACUREX CONTRACT NO. A81-05-030
RESULTS REPORT TO L. Waterland
TELEPHONE
ADDRESS
Ethan Allen - 2 SASS

SAMPLE ID (CUSTOMER)	Probe	1u	3u	10u	Filter	XAD				
SAMPLE ID (LAB)	671	674	673	672	681	676				
PARAMETER										UNITS
Weight	0.3089	0.7075	2.7042	4.1327	~~130~~					gram
					1.4901	130				

ANALYST J. Labash
REVIEWER G. Nicoll

Form EED-057 4/80

ISOKINETIC PERFORMANCE WORKSHEET & PARTICULATE CALCULATIONS

Plant ETHAN ALLEN
Performed by DAROS
Date 4-16-81
Sample Location STACK
Test No./Type 2 - SASS

Barometric Pressure (in. Hg)	P_b	28.975
Meter volume (std), $17.64\left(\frac{V_m}{\alpha}\right)\left(\frac{P_b + \frac{\Delta H}{13.6}}{T_m + 460}\right)$ $17.64\left(\frac{(1028)}{(1.007)}\right)\left(\frac{(28.975) + \frac{(-.3)}{13.6}}{(85.0) + 460}\right)$	V_m std	955.777
Volume of liquid collected (grams)	Vl_c	1781
Volume of liquid at standard condition (scf) $Vl_c \times 0.04707$	V_w std	83.83
Stack gas proportion of water vapor $\frac{V_{w\,std}}{V_{w\,std} + V_{m\,std}} = \frac{(83.83)}{(83.83) + (955.777)}$	B_{wo}	0.081
Molecular weight, stack gas dry (lb/lb-mole) $(\%\ CO_2 \times 0.44) + (\%\ O_2 \times 0.32) + (\%\ N_2 + \%\ CO \times 0.28)$ $(5.5 \times 0.44) + (15 \times 0.32) + (79.5 + \underline{\quad} \times 0.28)$	M_d	29.48
Molecular weight, stack gas wet (lb/lb-mole) $Md(1-B_{wo}) + 18(B_{wo}) = (29.48)(1-0.08) + 18(0.08)$	M_s	28.56
Absolute stack pressure (in. Hg) $P_b + \frac{P_{stack}\ (in.\ H_2O)}{13.6} = (28.975) + \frac{(-.3)}{13.6}$	P_s	28.95

7602/5/81/Rev 1

Temperature stack gas, average (^oF)	T_s	308
Stack velocity (fps) $85.49\ (C_p)\ (\sqrt{\Delta P}_{s\ avg})\ \sqrt{\frac{T_s avg + 460}{P_s\ \ M_s}}$ $85.49\ (\underline{0.79})(0.458)\ \sqrt{\frac{(\underline{308}) + 460}{(\underline{28.55})(\underline{28.56})}}$	$V_{s(avg)}$	29.81
Total sample time (minutes)	θ	270
Nozzle diameter, actual (inches)	N_d	0.741
Percent isokinetic (%) $\frac{17.33\ (T_s + 460)(V_w\ std + V_m\ std)}{\theta\ \ \ V_s\ \ \ P_s\ \ \ N_d^2}$ $\frac{17.33\ (\underline{308} + 460)((\underline{88.83}) + (\underline{255.28}))}{(\underline{270})(\underline{29.81})(\underline{28.55})(\underline{2,741})}$	%I	108.1
Area of stack (ft^2) $\pi = 3.1416$ $\pi r^2 \div 144$, $\pi(\underline{\quad})^2 \div 144$	A_s	17.57
Stack gas volume at standard conditions (dscfm) $60\ (1 - B_{wo})Vs_{avg}\ A_s \left(\frac{528}{T_s\ avg + 460}\right) \left(\frac{Ps}{29.92}\right)$ $60\ (1 - \underline{0.081})(\underline{29.8})(\underline{17.57})\left(\frac{528}{\underline{308} + 460}\right) \left(\frac{(\underline{28.95})}{(29.92)}\right)$	Q_s	13,744
Particulate matter concentration, dry (gr/dscf) $15.432\ \frac{M_p(grams)}{Vm_{std}}$, $15.432\ \frac{(\underline{9.3434})}{(\underline{255.277})}$	$C_{s(std)}$	0.1508
Emission rate of particulate matter (lb/hr) $0.00857\ (Q_s)\ C_{s(std)}$, $0.00857\ (\underline{13,741})(\underline{0.1528})$	E_p	17.7691

7602/5/81/Rev 1

5.3 PARTICULATE EMISSIONS FROM EPA METHOD 5 SAMPLES

ACUREX
ANALYTICAL REPORT

Sample of: Ethan Allen

Sample Date: April 15, 1981

Requested By: Bruce Daros

I.D. Number: 7735.12 / CMEA

Analytical Method: EPA Method 5 Protocol - Ether / Chloroform Extraction

Date of Analysis: September 3, 1981 of Impinger Liquids

Lab I.D. Number	Component	Analytical Result	Unit
813668 - Test 1	526 mls		Net Gain milligrams
- Aqueous Phase		5.77	
- Organic Phase		17.96	
813714 - Test 2	511 mls		
- Aqueous Phase		1.07	
- Organic Phase		6.99	

Analysis By J. Sayer / L. Whitmer

Date September 15, 1981

Form EED 082 5/81

ACUREX
ANALYTICAL REPORT

Sample of: Ethan Allen

Sample Date: ~~7~~ April 15, 1981

Requested By: Bruce Daros

I.D. Number: 7735.12/CMEA

Analytical Method: Gravimetric Analysis of Filters

Date of Analysis: July 30, 1981

Lab I.D. Number	Component	Analytical Result	Unit
813667 - Test 1	MV-142-210	1.25237 -1.01180 =24057	FINAL Filter Weight *
813713 - Test 2	MV-142-222	1.37173 -1.02060 = 351.13	

Blank Correction Value -.021 mg

*Note: Due to unavailability of tare weights for these filters the Net Gain is NOT reported but rather the filter's actual final weight.

Analysis By: J. Sayer ... Whitmer

Date: 9/15/81

Form EED 082 5/81

ACURREX

ANALYTICAL REPORT

Sample of: Ethan Allen

Sample Date: April 15, 1981

Requested By: Bruce Daros

I.D. Number: 773512/CMEA

Analytical Method: Gravimetric Analysis of Probe Wash (Acetone)

Date of Analysis: September 1 and 2, 1981

Lab I.D. Number	Component	Analytical Result	Unit
813669-Test 1 Probe wash	120 mls	58.30	Net Gain milligrams
813712-Test 2	120 mls	229.03	

Analysis By: J. Sayer/L. Whitmer

Date: September 16, 1981

7735.12

B.C. Du Ros

CMEA: ETHAN ALLEN
OLD FORT, N.C.

TEST I.D.	V_M (STD)	FRONT HALF		BACK HALF					
					IMPINGER CONTENTS				
		PROBE & NOZZEL CATCH (Mg) 1/	M-5 FILTER (Mg) 1/	IMPINGER RINSE (acetone) (Mg) 1/	AQUEOUS PHASE (Mg) 1/	ORGANIC PHASE (Mg) 1/			
M-5 TEST No 1	64.627	58.30	240.59	2/	5.77	17.96			
M-5 TEST No 2	63.470	229.03	351.15	2/	1.07	6.99			

1/ Blank corrected value

2/ Sample was not obtained

ISOKINETIC PERFORMANCE WORKSHEET & PARTICULATE CALCULATIONS

Plant ETHAN ALLEN

Performed by

Date 4-15-81

Sample Location STACK

Test No./Type 1-M5

Barometric Pressure (in. Hg)	P_b	28.92
Meter volume (std), $17.64 \left(\frac{V_m}{\alpha}\right)\left(\frac{P_b + \frac{\Delta H}{13.6}}{T_m + 460}\right)$ $17.64 \left(\frac{(66.824)}{(0.9928)}\right)\left(\frac{(28.92) + \frac{(1.09)}{13.6}}{(72.8) + 460}\right)$	$V_{m\ std}$	64.627
Volume of liquid collected (grams)	Vl_c	69.4
Volume of liquid at standard condition (scf) $Vl_c \times 0.04707$	$V_{w\ std}$	3.267
Stack gas proportion of water vapor $\frac{V_{w\ std}}{V_{w\ std} + V_{m\ std}}, \frac{(3.267)}{(3.267) + (64.627)}$	B_{wo}	0.0481
Molecular weight, stack gas dry (lb/lb-mole) $(\%\ CO_2 \times 0.44) + (\%\ O_2 \times 0.32) + (\%\ N_2 + \%\ CO \times 0.28)$ $(4.0 \times 0.44) + (16 \times 0.32) + (80 + — \times 0.28)$	M_d	29.28
Molecular weight, stack gas wet (lb/lb-mole) $Md(1-B_{wo}) + 18(B_{wo}), (29.28)(1-0.048) + 18(0.048)$	M_s	28.74
Absolute stack pressure (in. Hg) $P_b + \frac{P_{stack}\ (in.\ H_2O)}{13.6}, (28.92) + \frac{(-.3)}{13.6}$	P_s	28.90

7602/5/81/Rev 1

Temperature stack gas, average (^{0}F)	T_s	308
Stack velocity (fps) $85.49\ (C_p)\ (\sqrt{\Delta P}_{s\ avg})\ \sqrt{\frac{T_s avg + 460}{P_s\ \ M_s}}$ $85.49\ (\underline{.79})(\ 0.435)\ \sqrt{\frac{(\underline{308}) + 460}{(\underline{28.90})(\underline{28.79})}}$	$V_{s(avg)}$	28.25
Total sample time (minutes)	θ	120
Nozzle diameter, actual (inches)	N_d	0.3086
Percent isokinetic (%) $\frac{17.33\ (T_s + 460)(V_w\ std + V_m\ std)}{\theta\quad V_s\quad P_s\quad N_d^2}$ $\frac{17.33\ (\underline{308} + 460)((\underline{3.267}) + (\underline{64.627}))}{(\underline{120})(\underline{28.25})(\underline{28.90})(\underline{.3086}^2)}$	%I	96.85
Area of stack (ft^2) $\pi = 3.1416$ $\pi r^2 \div 144$, $\pi(\underline{\quad})^2 \div 144$	A_s	12.57
Stack gas volume at standard conditions (dscfm) $60\ (1 - B_{wo})Vs_{avg}\ A_s \left(\frac{528}{T_s\ avg + 460}\right) \left(\frac{Ps}{29.92}\right)$ $60\ (1 - \underline{.0481})(\underline{28.25})(\underline{12.57})\left(\frac{528}{\underline{308} + 460}\right) \left(\frac{(\underline{28.90})}{(29.92)}\right)$	Q_s	13,468
Particulate matter concentration, dry (gr/dscf) $15.432\ \frac{M_p(grams)}{Vm_{std}}$, $15.432\ \frac{(\underline{.2989})}{(\underline{64.627})}$	$C_{s(std)}$	0.0714 solid 0.0057 condensable
Emission rate of particulate matter (lb/hr) $0.00857\ (Q_s)\ C_{s(std)}$, $0.00857\ (\underline{13,468})(\underline{.0714})$ .005)	E_p	8.2410 solid 0.6579 condensable 8.8989 Total

ISOKINETIC PERFORMANCE WORKSHEET & PARTICULATE CALCULATIONS

Plant ETHAN ALLEN Performed by [illegible]

Date 4-16-81

Sample Location STACK

Test No./Type 2 - M5

Barometric Pressure (in. Hg)	P_b	28.97
Meter volume (std), $17.64 \left(\frac{V_m}{\alpha}\right)\left(\frac{P_b + \frac{\Delta H}{13.6}}{T_m + 460}\right)$ $17.64 \left(\frac{(65.52)}{(.9828)}\right)\left(\frac{(28.57) + \frac{(1.466)}{13.6}}{(77) + 460}\right)$	V_m std	63.47
Volume of liquid collected (grams)	Vl_c	124.5
Volume of liquid at standard condition (scf) $Vl_c \times 0.04707$	V_w std	5.86
Stack gas proportion of water vapor $\frac{V_w \text{ std}}{V_w \text{ std} + V_m \text{ std}} = \frac{(5.86)}{(5.86) + (63.47)}$	B_{wo}	0.08
Molecular weight, stack gas dry (lb/lb-mole) $(\% CO_2 \times 0.44) + (\% O_2 \times 0.32) + (\% N_2 + \% CO \times 0.28)$ $(5.5 \times 0.44) + (15.0 \times 0.32) + (79.5 + \text{—} \times 0.28)$	M_d	29.48
Molecular weight, stack gas wet (lb/lb-mole) $Md(1-B_{wo}) + 18(B_{wo}) = (29.48)(1-.08) + 18(.08)$	M_s	28.56
Absolute stack pressure (in. Hg) $P_b + \frac{P_{stack} \text{ (in. } H_2O)}{13.6} = (28.57) + \frac{(-.3)}{13.6}$	P_s	28.57

7602/5/81/Rev 1

Temperature stack gas, average (^oF)	T_s	304
Stack velocity (fps) $85.49\,(C_p)\,(\sqrt{\Delta P}_{s\ avg})\sqrt{\frac{T_s avg + 460}{P_s \quad M_s}}$ $85.49\,(.79)(\ 0.420)\sqrt{\frac{(304) + 460}{(28.77)(28.56)}}$	$V_{s(avg)}$	27.26
Total sample time (minutes)	θ	120
Nozzle diameter, actual (inches)	N_d	0.3086
Percent isokinetic (%) $\frac{17.33\ (T_s + 460)(V_w\ std + V_m\ std)}{\theta \quad V_s \quad P_s \quad N_d^2}$ $\frac{17.33\ (304 + 460)((5.86) + (63.47))}{(120)(27.26)(28.77)(.3086^2)}$	%I	101.7
Area of stack (ft^2) $\pi = 3.1416$ $\pi r^2 \div 144$, $\pi(\_\_\_\_)^2 \div 144$	A_s	12.57
Stack gas volume at standard conditions (dscfm) $60\ (1 - B_{wo})Vs_{avg}\ A_s \left(\frac{528}{T_s\ avg + 460}\right)\left(\frac{Ps}{29.92}\right)$ $60\ (1 - .08)(27.26)(12.57)\left(\frac{528}{304 + 460}\right)\left(\frac{(28.5)}{(29.92)}\right)$	Q_s	12656
Particulate matter concentration, dry (gr/dscf) $15.432\ \frac{M_p(grams)}{Vm_{std}}$, $15.432\ \frac{(.5802)}{(63.47)}$ .0081	$C_{s(std)}$	0.1411 solid 0.0020 condensable
Emission rate of particulate matter (lb/hr) $0.00857\ (Q_s)\ C_{s(std)}$, $0.00857\ (12656)(.1411)$ .0020	E_p	15.305 solid 0.217 condensable 15.522 Total

5.4 SULFUR OXIDE EMISSIONS FROM CONTROLLED CONDENSATION SAMPLES

CONTROLLED CONDENSATION SYSTEM (CCS)
LABORATORY DATA SHEET

Plant ETHAN ALLEN, OLD FORT, N.C. Analyst B.C. DraRos
Date 4-14-81 Date Lab Analysis Completed 10-20-81
Sample Location STACK
Run No. 1-CCC

Titration Data

Method BARIUM/THORIN Titrant $BACl_2$ Normality .0166 Indicator THORIN

Sample Description	Probe, Nozzle and Filter Rinse	G/R Coil Rinse	Impinger Contents and Rinse	H_2O Blank	3% H_2O_2 Blank
Sample No.	813639	813658	813637	813638	813635
Vol. of Sample	120.0	81.0	452.0	UNITY	UNITY
Vol. of Aliquot	10.0	10.0	10.0	10.0	10.0
Vol. of Titrant Used	.05 .05 —	.05 .05 —	.05 .05 —	.05 .05 —	.05 .05 —
Average Vol. of Titrant Used	.05	.05	.05	.05	.05

Calculations

Vol. of Gas Sampled (V_M) 19.535 ft^3, Avg. Meter Temp (T_M) 94 °F,
Meter Pressure (P_M) 28.91 "Hg, Meter α Factor 1.007 dimensionless

$$\text{PPM SO}_4 = \frac{48.15\ (\_\_\_,\ \text{MgSO}_4)(\_\_\_\_,\ T_M+460)}{96\ (\_\_\_\_\_\_\_,\ V_M)(\_\_\_\_\_\_\_\_,\ P_M)}\ (\_\_\_,\alpha)$$

ppm SO_4 = Ø

$$\text{PPM SO}_2 = \frac{48.15\ (\_\_\_,\ \text{MgSO}_2)(\_\_\_\_,\ T_M+460)}{64\ (\_\_\_\_\_\_\_,\ V_M)(\_\_\_\_\_\_\_\_,\ P_M)}\ (\_\_\_,\alpha)$$

ppm SO_2 = Ø

CONTROLLED CONDENSATION SYSTEM (CCS)
LABORATORY DATA SHEET

Plant ETHAN ALLEN, OLD FORT, N.C. Analyst B.C. DuBos
Date 4-16-81 Date Lab Analysis Completed 10-20-81
Sample Location STACK
Run No. 2-CCC

Titration Data

Method BARIUM/THORIN Titrant $BaCl_2$ Normality .0166 Indicator THORIN

Sample Description	Probe, Nozzle and Filter Rinse	G/R Coil Rinse	Impinger Contents and Rinse	H_2O Blank	3% H_2O_2 Blank
Sample No.	813750	813749	813748	813638	813663
Vol. of Sample	84.0	70.0	365.0	UNITY	UNITY
Vol. of Aliquot	10.0	10.0	10.0	10.0	10.0
Vol. of Titrant Used	.05 .05 —	.05 .05 —	.05 .05 —	.05 .05 —	.05 .05 —
Average Vol. of Titrant Used	.05	.05	.05	.05	.05

Calculations

Vol. of Gas Sampled (V_M) 19.320 ft^3, Avg. Meter Temp (T_M) 87 ^oF,
Meter Pressure (P_M) 28.91 "Hg, Meter α Factor 1.007 dimensionless

$$\text{PPM } SO_4 = \frac{48.15\ (\_\_\_,\ MgSO_4)(\_\_\_\_,\ T_M+460)}{96\ (\_\_\_\_\_\_\_,\ V_M)(\_\_\_\_\_\_\_\_,\ P_M)}\ (\_\_\_,\alpha)$$

ppm SO_4 = 0

$$\text{PPM } SO_2 = \frac{48.15\ (\_\_\_,\ MgSO_2)(\_\_\_\_,\ T_M+460)}{64\ (\_\_\_\_\_\_\_,\ V_M)(\_\_\_\_\_\_\_\_,\ P_M)}\ (\_\_\_,\alpha)$$

ppm SO_2 = 0

5.5 TRACE ELEMENTS AND LEACHABLE ANION ANALYSES

COMMERCIAL TESTING & ENGINEERING CO.
Reply to
GENERAL OFFICES: 228 NORTH LA SALLE STREET, CHICAGO, ILLINOIS 60601 · AREA CODE 312 726-8434
INSTRUMENTAL ANALYSIS DIVISION, 14335 WEST 44TH AVENUE, GOLDEN, COLORADO 80401, PHONE 303-278-9521

To: Mr. Roy A. Belletto
Acurex Corporation
485 Clyde Avenue
Mountain View, CA 94042

Date August 21, 1981

Release No. 5
P. O. No.: Subcontract SW59159A

Analyst: J. Oldham

Sample No.: A81-05-030-642
EA Filter Blank

SPARK SOURCE MASS SPECTROGRAPHIC ANALYSIS

IAD No. 97-G852-116-25

CONCENTRATION IN $\mu g/cm^2$

ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.
Uranium		Terbium		Ruthenium		Vanadium	0.006
Thorium		Gadolinium		Molybdenum	<0.001	Titanium	0.8
Bismuth		Europium		Niobium	0.002	Scandium	0.002
Lead	*0.04	Samarium		Zirconium	0.02	Calcium	MC
Thallium		Neodymium	<0.001	Yttrium	0.003	Potassium	0.06
Mercury	NR	Praseodymium	<0.001	Strontium	0.02	Chlorine	0.07
Gold		Cerium	0.007	Rubidium	<0.001	Sulfur	0.03
Platinum		Lanthanum	0.008	Bromine	0.07	Phosphorus	0.1
Iridium		Barium	0.1	Selenium		Silicon	MC
Osmium		Cesium		Arsenic	NR	Aluminum	>0.3
Rhenium		Iodine	0.001	Germanium		Magnesium	*MC
Tungsten		Tellurium		Gallium	0.003	Sodium	* >0.8
Tantalum		Antimony	NR	Zinc	0.08	Fluorine	≈1
Hafnium		Tin	<0.001	Copper	0.009	Oxygen	NR
Lutetium		Indium	STD	Nickel	0.005	Nitrogen	NR
Ytterbium		Cadmium		Cobalt	0.002	Carbon	NR
Thulium		Silver		Iron	0.3	Boron	2
Erbium		Palladium		Manganese	0.007	Beryllium	
Holmium		Rhodium		Chromium	0.009	Lithium	0.002
Dysprosium		*Heterogeneous				Hydrogen	NR

STD — Internal Standard
NR — Not Reported
All elements not detected < $0.001 \mu g/cm^2$
MC — Major Component > $10 \mu g/cm^2$
INT — Interference

Approved: M.L. Jacobs by [illegible]
24 Aug 81

COMMERCIAL TESTING & ENGINEERING CO.
GENERAL OFFICES: 228 NORTH LA SALLE STREET, CHICAGO, ILLINOIS 60601 · AREA CODE 312 726-8434
Reply to INSTRUMENTAL ANALYSIS DIVISION, 14335 WEST 44TH AVENUE, GOLDEN, COLORADO 80401, PHONE 303-278-9521

To: Mr. Roy A. Belletto
Acurex Corporation
485 Clyde Avenue
Mountain View,CA 94942

Date August 20, 1981

Release No. 5

Analyst: J. Oldham

P. O. No.: Subcontract SW59159A

Sample No.:A81-05-030-651
EA XAD Blank

IAD No. 97-G852-116-25

SPARK SOURCE MASS SPECTROGRAPHIC ANALYSIS

CONCENTRATION IN PPM WEIGHT

ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.
Uranium	≤0.3	Terbium		Ruthenium		Vanadium	<0.1
Thorium		Gadolinium		Molybdenum	0.4	Titanium	6
Bismuth		Europium		Niobium		Scandium	
Lead	0.4	Samarium		Zirconium	0.2	Calcium	37
Thallium		Neodymium		Yttrium		Potassium	24
Mercury	NR	Praseodymium		Strontium	<0.1	Chlorine	7
Gold		Cerium		Rubidium	<0.1	Sulfur	6
Platinum	*4	Lanthanum		Bromine	0.3	Phosphorus	4
Iridium		Barium	0.5	Selenium		Silicon	17
Osmium		Cesium	<0.1	Arsenic	NR	Aluminum	1
Rhenium		Iodine	<0.1	Germanium		Magnesium	2
Tungsten		Tellurium		Gallium	0.1	Sodium	4
Tantalum		Antimony	NR	Zinc	3	Fluorine	=0.4
Hafnium		Tin		Copper	3	Oxygen	NR
Lutetium		Indium	STD	Nickel	8	Nitrogen	NR
Ytterbium		Cadmium		Cobalt	<0.1	Carbon	NR
Thulium		Silver	<0.1	Iron	12	Boron	<0.1
Erbium		Palladium		Manganese	0.6	Beryllium	
Holmium		Rhodium		Chromium	*2	Lithium	<0.1
Dysprosium						Hydrogen	NR

*Heterogeneous

STD — Internal Standard
NR — Not Reported
All elements not detected < 0.1ppm
MC — Major Component
INT — Interference

Approved: M. L. Jacobs by PLSayler
24 Aug 81

COMMERCIAL TESTING & ENGINEERING CO.
GENERAL OFFICES: 228 NORTH LA SALLE STREET, CHICAGO, ILLINOIS 60601 · AREA CODE 312 726-8434
Reply to INSTRUMENTAL ANALYSIS DIVISION, 14335 WEST 44TH AVENUE, GOLDEN, COLORADO 80401, PHONE 303-278-9521

To: Mr. Roy A. Belletto
Acurex Corporation
485 Clyde Avenue
Mountain View, CA 94942

Date August 20, 1981

Release No 5
P. O. No.: Subcontract SW59159A

Analyst: J. Oldham

Sample No.: A81-05-030-654
EA Imp 1 Blank

SPARK SOURCE MASS SPECTROGRAPHIC ANALYSIS
CONCENTRATION IN µg/ml

IAD No.: 97-G852-116-25

ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.
Uranium		Terbium		Ruthenium		Vanadium	0.003
Thorium		Gadolinium		Molybdenum	0.008	Titanium	0.04
Bismuth		Europium		Niobium		Scandium	<0.001
Lead	0.003	Samarium		Zirconium	0.001	Calcium	0.5
Thallium		Neodymium		Yttrium		Potassium	0.1
Mercury	NR	Praseodymium		Strontium	<0.001	Chlorine	0.04
Gold		Cerium		Rubidium		Sulfur	0.1
Platinum		Lanthanum		Bromine	*0.05	Phosphorus	0.1
Iridium		Barium	0.008	Selenium		Silicon	0.6
Osmium		Cesium		Arsenic	NR	Aluminum	0.04
Rhenium		Iodine	0.002	Germanium		Magnesium	0.03
Tungsten		Tellurium		Gallium		Sodium	0.6
Tantalum		Antimony	NR	Zinc	0.02	Fluorine	≈0.7
Hafnium		Tin	0.02	Copper	0.005	Oxygen	NR
Lutetium		Indium	STD	Nickel	0.05	Nitrogen	NR
Ytterbium		Cadmium		Cobalt	0.003	Carbon	NR
Thulium		Silver		Iron	0.01	Boron	<0.001
Erbium		Palladium		Manganese	<0.001	Beryllium	
Holmium		Rhodium		Chromium	0.004	Lithium	0.01
Dysprosium						Hydrogen	NR

*Heterogeneous

STD — Internal Standard
NR — Not Reported
All elements not detected < 0.001µg/ml
MC — Major Component > 10µg/ml
INT — Interference

Approved: M.L. Jacobs by [illegible]
24 Aug 81

COMMERCIAL TESTING & ENGINEERING CO.
Reply to
GENERAL OFFICES: 228 NORTH LA SALLE STREET, CHICAGO, ILLINOIS 60601 · AREA CODE 312 726-8434
INSTRUMENTAL ANALYSIS DIVISION, 14335 WEST 44TH AVENUE, GOLDEN, COLORADO 80401, PHONE 303-278-9521

To: Mr. Roy A. Belletto
Acurex Corporation
485 Clyde Avenue
Mountain View, CA 94942

Date August 25, 1981

Release No. 5
P. O. No.: Subcontract SW59159A

Analyst: J. Oldham

Sample No.: A81-05-030-661
EA-1 fuel

SPARK SOURCE MASS SPECTROGRAPHIC ANALYSIS

IAD No.: 97-G852-116-25

CONCENTRATION IN PPM WEIGHT

ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.
Uranium	<0.03	Terbium		Ruthenium		Vanadium	0.08
Thorium	<0.04	Gadolinium		Molybdenum	≤0.01	Titanium	0.05
Bismuth		Europium		Niobium	<0.01	Scandium	
Lead	0.4	Samarium	≤0.02	Zirconium	0.07	Calcium	MC
Thallium	0.03	Neodymium	≤0.01	Yttrium	0.04	Potassium	>54
Mercury	NR	Praseodymium	0.02	Strontium	7	Chlorine	10
Gold		Cerium	0.1	Rubidium	0.4	Sulfur	>27
Platinum		Lanthanum	0.2	Bromine	0.4	Phosphorus	19
Iridium		Barium	21	Selenium	0.01	Silicon	MC
Osmium		Cesium	0.06	Arsenic	NR	Aluminum	>4
Rhenium		Iodine	0.09	Germanium		Magnesium	MC
Tungsten		Tellurium	0.03	Gallium	0.01	Sodium	>11
Tantalum		Antimony	NR	Zinc	29	Fluorine	≈0.6
Hafnium		Tin	<0.01	Copper	6	Oxygen	NR
Lutetium		Indium	STD	Nickel	0.08	Nitrogen	NR
Ytterbium		Cadmium	0.03	Cobalt	0.1	Carbon	NR
Thulium		Silver	0.2	Iron	11	Boron	*0.04
Erbium		Palladium		Manganese	>45	Beryllium	
Holmium		Rhodium		Chromium	0.1	Lithium	0.03
Dysprosium						Hydrogen	NR

*Heterogeneous

Note: Sample low temperature oxygen plasma ashed prior to analysis.

STD — Internal Standard
NR — Not Reported
All elements not detected < 0.01ppm
MC — Major Component >100ppm
INT — Interference

Approved: M L Jacobs

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COMMERCIAL TESTING & ENGINEERING CO.
GENERAL OFFICES: 228 NORTH LA SALLE STREET, CHICAGO, ILLINOIS 60601 · AREA CODE 312 726-8434
Reply to INSTRUMENTAL ANALYSIS DIVISION, 14335 WEST 44TH AVENUE, GOLDEN, COLORADO 80401, PHONE 303-278-9521

To: Mr. Roy A. Belletto
Acurex Corporation
485 Clyde Avenue
Mountain View, CA 94942

Date August 19, 1981

Analyst J.Oldham

Release No. 5
P. O. No.: Subcontract No. SW59159A
Sample No.: A81-05-030-646
EA-1 10μ + 3μ

IAD No. 97-G852-116-25

SPARK SOURCE MASS SPECTROGRAPHIC ANALYSIS
CONCENTRATION IN PPM WEIGHT

ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.
Uranium	1	Terbium	1	Ruthenium		Vanadium	17
Thorium	4	Gadolinium	1	Molybdenum	10	Titanium	MC
Bismuth		Europium	0.5	Niobium	5	Scandium	0.5
Lead	41	Samarium	5	Zirconium	6	Calcium	MC
Thallium		Neodymium	4	Yttrium	7	Potassium	MC
Mercury	NR	Praseodymium	2	Strontium	MC	Chlorine	680
Gold		Cerium	13	Rubidium	79	Sulfur	MC
Platinum		Lanthanum	42	Bromine	8	Phosphorus	MC
Iridium		Barium	MC	Selenium	0.5	Silicon	MC
Osmium		Cesium	0.6	Arsenic	NR	Aluminum	MC
Rhenium		Iodine	1	Germanium	0.5	Magnesium	MC
Tungsten	5	Tellurium	0.3	Gallium	7	Sodium	MC
Tantalum		Antimony	NR	Zinc	MC	Fluorine	*MC
Hafnium		Tin	0.4	Copper	98	Oxygen	NR
Lutetium	0.1	Indium	STD	Nickel	17	Nitrogen	NR
Ytterbium	0.9	Cadmium	0.7	Cobalt	2	Carbon	NR
Thulium	0.1	Silver	4	Iron	MC	Boron	190
Erbium	0.4	Palladium		Manganese	MC	Beryllium	<0.1
Holmium	0.5	Rhodium		Chromium	26	Lithium	3
Dysprosium	2	*Heterogeneous				Hydrogen	NR

STD — Internal Standard
NR — Not Reported
All elements not detected < 0.1ppm
MC — Major Component
INT — Interference

Approved: M. L. Jacobs by PLStaylor
24 Aug '81

COMMERCIAL TESTING & ENGINEERING CO.
GENERAL OFFICES: 228 NORTH LA SALLE STREET, CHICAGO, ILLINOIS 60601 · AREA CODE 312 726-8434
Reply to INSTRUMENTAL ANALYSIS DIVISION, 14335 WEST 44TH AVENUE, GOLDEN, COLORADO 80401, PHONE 303-278-9521

To: Mr. Roy A. Belletto
Acurex Corporation
485 Clyde Avenue
Mountain View, CA 94942

Date August 20, 1981

Release No. 5

Analyst: J. Oldham

P. O. No.: Subcontract No. SW59159A

Sample No.: A81-05-030-644
EA-1 1μ + filter

IAD No.: 97-G852-116-25

SPARK SOURCE MASS SPECTROGRAPHIC ANALYSIS

CONCENTRATION IN μg/cm²

ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.
Uranium	<0.001	Terbium	0.001	Ruthenium		Vanadium	0.008
Thorium	0.002	Gadolinium	0.003	Molybdenum	0.005	Titanium	0.5
Bismuth	<0.001	Europium	0.001	Niobium	0.001	Scandium	0.001
Lead	0.2	Samarium	0.009	Zirconium	0.02	Calcium	MC
Thallium		Neodymium	0.01	Yttrium	0.03	Potassium	MC
Mercury	NR	Praseodymium	0.01	Strontium	2	Chlorine	1
Gold		Cerium	0.06	Rubidium	0.6	Sulfur	MC
Platinum		Lanthanum	0.1	Bromine	0.02	Phosphorus	MC
Iridium		Barium	MC	Selenium	0.03	Silicon	MC
Osmium		Cesium	0.002	Arsenic	NR	Aluminum	MC
Rhenium		Iodine	0.002	Germanium	0.002	Magnesium	MC
Tungsten	0.006	Tellurium		Gallium	0.01	Sodium	MC
Tantalum	0.002	Antimony	NR	Zinc	4	Fluorine	≈0.4
Hafnium		Tin	0.002	Copper	0.3	Oxygen	NR
Lutetium	<0.001	Indium	STD	Nickel	0.04	Nitrogen	NR
Ytterbium	0.001	Cadmium	0.002	Cobalt	0.003	Carbon	NR
Thulium	<0.001	Silver	0.04	Iron	MC	Boron	0.04
Erbium	<0.001	Palladium		Manganese	MC	Beryllium	<0.001
Holmium	0.001	Rhodium		Chromium	0.05	Lithium	<0.001
Dysprosium	0.002					Hydrogen	NR

STD — Internal Standard
NR — Not Reported
All elements not detected < 0.001μg/cm²
MC — Major Component > 10μg/cm²
INT — Interference

Approved: M.L. Jacobs by PK Taylor
24 Aug '81

COMMERCIAL TESTING & ENGINEERING CO.
GENERAL OFFICES: 228 NORTH LA SALLE STREET, CHICAGO, ILLINOIS 60601 · AREA CODE 312 726-8434
Reply to INSTRUMENTAL ANALYSIS DIVISION, 14335 WEST 44TH AVENUE, GOLDEN, COLORADO 80401, PHONE 303-278-9521

To: Mr. Roy A. Belletto
Acurex Corporation
485 Clyde Avenue
Mountain View, CA 94942

Date August 20, 1981

Release No. 5
P. O. No.: Subcontract No. SW59159A

Analyst J. Oldham

Sample No. A-81-05-030-650
EA-1 XAD

IAD No 97-G852-116-25

SPARK SOURCE MASS SPECTROGRAPHIC ANALYSIS

CONCENTRATION IN PPM WEIGHT

ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC
Uranium		Terbium		Ruthenium		Vanadium	<0.1
Thorium		Gadolinium		Molybdenum	0.4	Titanium	2
Bismuth		Europium		Niobium		Scandium	<0.1
Lead	0.5	Samarium		Zirconium	2	Calcium	25
Thallium		Neodymium		Yttrium		Potassium	46
Mercury	NR	Praseodymium		Strontium	<0.1	Chlorine	34
Gold		Cerium		Rubidium		Sulfur	160
Platinum	*2	Lanthanum	≤2	Bromine	2	Phosphorus	3
Iridium		Barium	0.4	Selenium		Silicon	11
Osmium		Cesium	0.3	Arsenic	NR	Aluminum	2
Rhenium		Iodine	*0.2	Germanium		Magnesium	*8
Tungsten		Tellurium		Gallium	<0.1	Sodium	64
Tantalum		Antimony	NR	Zinc	5	Fluorine	≈0.3
Hafnium		Tin		Copper	1	Oxygen	NR
Lutetium		Indium	STD	Nickel	27	Nitrogen	NR
Ytterbium		Cadmium		Cobalt	1	Carbon	NR
Thulium		Silver	130	Iron	18	Boron	<0.1
Erbium		Palladium		Manganese	0.4	Beryllium	
Holmium		Rhodium		Chromium	0.9	Lithium	0.1
Dysprosium		*Heterogeneous				Hydrogen	NR

STD — Internal Standard
NR — Not Reported
All elements not detected < 0.1ppm
MC — Major Component
INT — Interference

Approved: M.L. Jacobs by PCTaylor
24 Aug '81

COMMERCIAL TESTING & ENGINEERING CO.
GENERAL OFFICES: 228 NORTH LA SALLE STREET, CHICAGO, ILLINOIS 60601 · AREA CODE 312 726-8434
Reply to INSTRUMENTAL ANALYSIS DIVISION, 14335 WEST 44TH AVENUE, GOLDEN, COLORADO 80401 PHONE 303-278-9521

To: Mr. Roy A. Belletto
Acurex Corporation
485 Clyde Avenue
Mountain View, CA 94042

Date September 2, 1981

Release No. 5
P. O. No.: Subcontract SW 59159A

Analyst J. Oldham

Sample No. A81-05-030-652
EA-1 Imp 1

SPARK SOURCE MASS SPECTROGRAPHIC ANALYSIS
CONCENTRATION IN PPM WEIGHT

IAD No. 97-G852-116-25

ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.
Uranium		Terbium		Ruthenium		Vanadium	0.007
Thorium		Gadolinium		Molybdenum	0.2	Titanium	0.1
Bismuth		Europium		Niobium	0.005	Scandium	≤0.002
Lead		Samarium		Zirconium	0.03	Calcium	0.4
Thallium		Neodymium		Yttrium		Potassium	*0.6
Mercury	NR	Praseodymium		Strontium	0.004	Chlorine	0.4
Gold		Cerium		Rubidium	0.08	Sulfur	MC
Platinum		Lanthanum		Bromine	0.08	Phosphorus	0.09
Iridium		Barium	0.05	Selenium		Silicon	MC
Osmium		Cesium		Arsenic	NR	Aluminum	0.08
Rhenium		Iodine		Germanium		Magnesium	0.7
Tungsten		Tellurium		Gallium	<0.001	Sodium	MC
Tantalum		Antimony	NR	Zinc	*1	Fluorine	≈3
Hafnium		Tin		Copper	0.01	Oxygen	NR
Lutetium		Indium	STD	Nickel	0.08	Nitrogen	NR
Ytterbium		Cadmium		Cobalt		Carbon	NR
Thulium		Silver	0.2	Iron	0.1	Boron	0.01
Erbium		Palladium		Manganese	0.005	Beryllium	
Holmium		Rhodium		Chromium	0.2	Lithium	<0.001
Dysprosium						Hydrogen	NR

* Heterogeneous

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SEP 01 1981

STD — Internal Standard
NR — Not Reported
All elements not detected <0.002 μg/ml
MC — Major Component >10μg/ml
INT — Interference

Approved: M J Jacobs

COMMERCIAL TESTING & ENGINEERING CO.
GENERAL OFFICES: 228 NORTH LA SALLE STREET, CHICAGO, ILLINOIS 60601 · AREA CODE 312 726-8434
Reply to INSTRUMENTAL ANALYSIS DIVISION, 14335 WEST 44TH AVENUE, GOLDEN, COLORADO 80401, PHONE 303-278-9521

To: Mr. Roy A. Belletto
Acurex Corporation
485 Clyde Avenue
Mountain View, CA 94942

Date August 20, 1981

Release No. 5

Analyst J. Oldham

P. O. No.: Subcontract SW59159A

Sample No.: A81-05-030-662
EA-1 flyash

IAD No. 97-G852-116-25

SPARK SOURCE MASS SPECTROGRAPHIC ANALYSIS
CONCENTRATION IN PPM WEIGHT

ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.
Uranium	1	Terbium	0.9	Ruthenium		Vanadium	29
Thorium	7	Gadolinium	2	Molybdenum	2	Titanium	MC
Bismuth		Europium	0.4	Niobium	4	Scandium	0.9
Lead	28	Samarium	3	Zirconium	10	Calcium	MC
Thallium		Neodymium	4	Yttrium	11	Potassium	MC
Mercury	NR	Praseodymium	4	Strontium	620	Chlorine	870
Gold		Cerium	20	Rubidium	130	Sulfur	MC
Platinum		Lanthanum	16	Bromine	6	Phosphorus	MC
Iridium		Barium	MC	Selenium	5	Silicon	MC
Osmium		Cesium	0.6	Arsenic	NR	Aluminum	MC
Rhenium		Iodine	0.7	Germanium	0.3	Magnesium	MC
Tungsten	0.9	Tellurium	0.5	Gallium	5	Sodium	MC
Tantalum		Antimony	NR	Zinc	410	Fluorine	24
Hafnium	0.9	Tin	0.5	Copper	52	Oxygen	NR
Lutetium		Indium	STD	Nickel	6	Nitrogen	NR
Ytterbium		Cadmium	1	Cobalt	3	Carbon	NR
Thulium		Silver		Iron	MC	Boron	140
Erbium	0.6	Palladium		Manganese	MC	Beryllium	0.3
Holmium	0.8	Rhodium		Chromium	4	Lithium	5
Dysprosium	1					Hydrogen	NR

STD — Internal Standard
NR — Not Reported
All elements not detected < 0.1ppm
MC — Major Component
INT — Interference

Approved: M. L. Jacobs by RF Saylor
24 Aug 81

COMMERCIAL TESTING & ENGINEERING CO.
GENERAL OFFICES: 228 NORTH LA SALLE STREET, CHICAGO, ILLINOIS 60601 · AREA CODE 312 726-8434
Reply to INSTRUMENTAL ANALYSIS DIVISION, 14335 WEST 44TH AVENUE, GOLDEN, COLORADO 80401, PHONE 303-278-9521

To: Mr. Roy A. Belletto
Acurex Corporation
485 Clyde Avenue
Mountain View,CA 94042

Date October 12, 1981

Release No. 6 Exhibit A
P. O. No.: Subcontract No. SW59159A

Analyst J. Oldham

Sample No.: A81-07-033-1
EA Flyash Leachate

SPARK SOURCE MASS SPECTROGRAPHIC ANALYSIS
CONCENTRATION IN μg/mL

IAD No. 97-H437-116-13

ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.
Uranium		Terbium		Ruthenium		Vanadium	0.08
Thorium		Gadolinium		Molybdenum	0.003	Titanium	2
Bismuth		Europium		Niobium		Scandium	<0.001
Lead	0.08	Samarium		Zirconium	0.002	Calcium	MC
Thallium		Neodymium		Yttrium	<0.001	Potassium	MC
Mercury	NR	Praseodymium		Strontium	MC	Chlorine	MC
Gold		Cerium	0.002	Rubidium	MC	Sulfur	MC
Platinum	≤0.005	Lanthanum	0.003	Bromine	0.4	Phosphorus	0.2
Iridium		Barium	MC	Selenium		Silicon	MC
Osmium		Cesium		Arsenic	NR	Aluminum	0.1
Rhenium		Iodine	0.05	Germanium	<0.001	Magnesium	0.05
Tungsten	0.09	Tellurium	≤0.008	Gallium	0.003	Sodium	MC
Tantalum	0.009	Antimony	NR	Zinc	0.02	Fluorine	≈4
Hafnium		Tin	≤0.009	Copper	0.03	Oxygen	NR
Lutetium		Indium	STD	Nickel	0.02	Nitrogen	NR
Ytterbium		Cadmium	0.002	Cobalt	≤0.002	Carbon	NR
Thulium		Silver		Iron	6	Boron	0.01
Erbium		Palladium		Manganese	0.005	Beryllium	
Holmium		Rhodium		Chromium	0.1	Lithium	0.01
Dysprosium						Hydrogen	NR

STD — Internal Standard
NR — Not Reported
All elements not detected < 0.001 μg/mL
MC — Major Component > 10 μg/mL
INT — Interference

Approved: M Jacobs

ACUREX Corporation
ANALYSIS LABORATORIES

DATA REPORTING FORM

CUSTOMER CMEA
DATE
CUSTOMER CONTRACT NO. 307736.12
ACUREX CONTRACT NO. A81-07-033
RESULTS REPORT TO L. Waterland
TELEPHONE
ADDRESS
Ethan Allen fly ash

SAMPLE ID (CUSTOMER)	662		662							
SAMPLE ID (LAB)	033-1	Blank	033-1							
PARAMETER										UNITS
F^-	0.2	<0.1	0.8							
CL^-	140	<5	560							
Br^-	10	<1	40							
NO_3^-	25	<0.1	100							
NO_2^-	59	<0.1	240							
$SO_3^=$	<2	<2	<8							
$SO_4^=$	200	<5	800							
$PO_4^{\equiv}$ as P	0.04	<0.02	0.2							
NH_4^+ as N	1.2	<0.5	5							
Units	mg/l	mg/l	mg/kg							

ANALYST

REVIEWER

Form EED 051 4-80

COMMERCIAL TESTING & ENGINEERING CO.
GENERAL OFFICES: 228 NORTH LA SALLE STREET, CHICAGO, ILLINOIS 60601 · AREA CODE 312 726-8434
Reply to INSTRUMENTAL ANALYSIS DIVISION, 14335 WEST 44TH AVENUE, GOLDEN, COLORADO 80401, PHONE 303-278-9521

To: Mr. Roy A. Belletto
Acurex Corporation
485 Clyde Avenue
Mountain View, CA 94942

Date August 25, 1981

Release No. 5
P. O. No.: Subcontract SW59159A

Analyst: J. Oldham

Sample No.: A81-05-031-743
EA-2 fuel

IAD No.: 97-G852-116-25

SPARK SOURCE MASS SPECTROGRAPHIC ANALYSIS

CONCENTRATION IN PPM WEIGHT

ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.
Uranium		Terbium		Ruthenium		Vanadium	0.6
Thorium		Gadolinium		Molybdenum	0.07	Titanium	9
Bismuth		Europium		Niobium	0.1	Scandium	0.01
Lead	0.3	Samarium		Zirconium	0.5	Calcium	MC
Thallium	0.04	Neodymium	0.02	Yttrium	0.06	Potassium	>92
Mercury	NR	Praseodymium	0.04	Strontium	12	Chlorine	4
Gold		Cerium	0.2	Rubidium	3	Sulfur	>47
Platinum		Lanthanum	0.2	Bromine	0.07	Phosphorus	MC
Iridium		Barium	36	Selenium	≤0.02	Silicon	MC
Osmium		Cesium	0.03	Arsenic	NR	Aluminum	>7
Rhenium		Iodine	0.04	Germanium	<0.01	Magnesium	MC
Tungsten		Tellurium	0.05	Gallium	0.1	Sodium	>19
Tantalum		Antimony	NR	Zinc	22	Fluorine	>7
Hafnium	0.08	Tin	0.03	Copper	3	Oxygen	NR
Lutetium		Indium	STD	Nickel	0.3	Nitrogen	NR
Ytterbium		Cadmium	0.1	Cobalt	0.5	Carbon	NR
Thulium		Silver	0.08	Iron	MC	Boron	0.03
Erbium		Palladium		Manganese	>76	Beryllium	<0.01
Holmium		Rhodium		Chromium	0.04	Lithium	0.05
Dysprosium						Hydrogen	NR

Note: Sample low temperature oxygen plasma ashed prior to analysis

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STD — Internal Standard
NR — Not Reported
All elements not detected < 0.01ppm
MC — Major Component >100ppm
INT — Interference

Approved: M L Jacobs

COMMERCIAL TESTING & ENGINEERING CO.

GENERAL OFFICES 228 NORTH LA SALLE STREET, CHICAGO ILLINOIS 60601 · AREA CODE 312 726-8434

Reply to INSTRUMENTAL ANALYSIS DIVISION 14335 WEST 44TH AVENUE GOLDEN, COLORADO 80401 PHONE 303-278-9521

To: Mr. Roy A. Belletto
Acurex Corporation
485 Clyde Avenue
Mountain View, CA 94942

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Date August 21, 1981

Release No. 5
P. O. No.: Subcontract SW59159A

Analyst: J. Oldham

Sample No. A-81-05-030-672
EA-2 10μ+ 3μ

SPARK SOURCE MASS SPECTROGRAPHIC ANALYSIS

CONCENTRATION IN PPM WEIGHT

IAD No. 97-G852-116-25

ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.
Uranium	2	Terbium	0.7	Ruthenium		Vanadium	29
Thorium	3	Gadolinium	2	Molybdenum	4	Titanium	MC
Bismuth	0.3	Europium	0.7	Niobium	6	Scandium	0.9
Lead	63	Samarium	3	Zirconium	*22	Calcium	MC
Thallium		Neodymium	4	Yttrium	13	Potassium	MC
Mercury	NR	Praseodymium	8	Strontium	MC	Chlorine	880
Gold		Cerium	41	Rubidium	300	Sulfur	MC
Platinum		Lanthanum	72	Bromine	14	Phosphorus	MC
Iridium		Barium	MC	Selenium	2	Silicon	MC
Osmium		Cesium	1	Arsenic	NR	Aluminum	MC
Rhenium		Iodine	1	Germanium	0.4	Magnesium	MC
Tungsten		Tellurium		Gallium	5	Sodium	MC
Tantalum		Antimony	NR	Zinc	460	Fluorine	≈55
Hafnium	*2	Tin	1	Copper	84	Oxygen	NR
Lutetium	0.2	Indium	STD	Nickel	6	Nitrogen	NR
Ytterbium	2	Cadmium	3	Cobalt	3	Carbon	NR
Thulium	0.2	Silver	≤1	Iron	MC	Boron	150
Erbium	0.6	Palladium		Manganese	MC	Beryllium	0.3
Holmium	0.8	Rhodium		Chromium	8	Lithium	22
Dysprosium	3	*Heterogeneous				Hydrogen	NR

STD — Internal Standard
NR — Not Reported
All elements not detected < 0.1ppm
MC — Major Component
INT — Interference

Approved M. L. Jacobs by [illegible]
24 Aug '81

COMMERCIAL TESTING & ENGINEERING CO.
GENERAL OFFICES: 228 NORTH LA SALLE STREET, CHICAGO, ILLINOIS 60601 · AREA CODE 312 726-8434
Reply to INSTRUMENTAL ANALYSIS DIVISION, 14335 WEST 44TH AVENUE, GOLDEN, COLORADO 80401, PHONE 303-278-9521

To: Mr. Roy A. Belletto
Acurex Corporation
485 Clyde Avenue
Mountain View, CA 94942

Date August 20, 1981

Analyst J. Oldham

Release No. 5
P. O. No.: Subcontract SW 59159A
Sample No.: A81-05-030-674
EA-2 1μ+ filter

IAD No.: 97-G852-116-25

SPARK SOURCE MASS SPECTROGRAPHIC ANALYSIS
CONCENTRATION IN μg/cm2

ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.
Uranium	0.002	Terbium	<0.001	Ruthenium		Vanadium	0.009
Thorium	0.002	Gadolinium	<0.001	Molybdenum	0.005	Titanium	0.6
Bismuth	0.004	Europium	<0.001	Niobium	0.001	Scandium	<0.001
Lead	0.2	Samarium	0.004	Zirconium	0.03	Calcium	MC
Thallium	<0.001	Neodymium	0.005	Yttrium	0.004	Potassium	>0.6
Mercury	NR	Praseodymium	0.002	Strontium	0.8	Chlorine	0.3
Gold		Cerium	0.01	Rubidium	0.3	Sulfur	>0.3
Platinum		Lanthanum	0.02	Bromine	0.02	Phosphorus	>2
Iridium		Barium	MC	Selenium	0.01	Silicon	>3
Osmium		Cesium	<0.001	Arsenic	NR	Aluminum	>0.05
Rhenium		Iodine	0.002	Germanium	0.001	Magnesium	>4
Tungsten	0.005	Tellurium		Gallium	0.01	Sodium	>0.1
Tantalum	<0.001	Antimony	NR	Zinc	6	Fluorine	≈0.2
Hafnium	<0.001	Tin	0.004	Copper	0.2	Oxygen	NR
Lutetium	<0.001	Indium	STD	Nickel	0.04	Nitrogen	NR
Ytterbium	<0.001	Cadmium	0.002	Cobalt	0.002	Carbon	NR
Thulium	<0.001	Silver	0.02	Iron	MC	Boron	>0.9
Erbium	<0.001	Palladium		Manganese	>0.5	Beryllium	<0.001
Holmium	<0.001	Rhodium		Chromium	0.02	Lithium	0.007
Dysprosium	<0.001					Hydrogen	NR

STD — Internal Standard
NR — Not Reported
All elements not detected < 0.001μg/cm2
MC — Major Component > 10μg/cm2
INT — Interference

Approved: M. L. Jacobs by
24 Aug '81

COMMERCIAL TESTING & ENGINEERING CO.
GENERAL OFFICES: 228 NORTH LA SALLE STREET, CHICAGO, ILLINOIS 60601 · AREA CODE 312 726-8434
Reply to INSTRUMENTAL ANALYSIS DIVISION 14335 WEST 44TH AVENUE, GOLDEN, COLORADO 80401, PHONE 303-278-9521

To: Mr. Roy A. Belletto
Acurex Corporation
485 Clyde Avenue
Mountain View, CA 94942

Date August 20, 1981

Release No. 5

Analyst J. Oldham

P. O. No.: Subcontract SW59159A

Sample No.: A81-05-030-676
EA-2 XAD

IAD No. 97-G852-116-25

SPARK SOURCE MASS SPECTROGRAPHIC ANALYSIS

CONCENTRATION IN PPM WEIGHT

ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.
Uranium		Terbium		Ruthenium		Vanadium	<0.1
Thorium		Gadolinium		Molybdenum	1	Titanium	3
Bismuth		Europium		Niobium		Scandium	
Lead	0.4	Samarium		Zirconium	0.5	Calcium	180
Thallium		Neodymium		Yttrium		Potassium	130
Mercury	NR	Praseodymium		Strontium	0.2	Chlorine	5
Gold		Cerium		Rubidium		Sulfur	7
Platinum	2	Lanthanum		Bromine	2	Phosphorus	4
Iridium		Barium	0.8	Selenium		Silicon	15
Osmium		Cesium		Arsenic	NR	Aluminum	12
Rhenium		Iodine	0.1	Germanium		Magnesium	4
Tungsten		Tellurium		Gallium		Sodium	12
Tantalum		Antimony	NR	Zinc	25	Fluorine	≈3
Hafnium		Tin		Copper	10	Oxygen	NR
Lutetium		Indium	STD	Nickel	67	Nitrogen	NR
Ytterbium		Cadmium		Cobalt	≤0.1	Carbon	NR
Thulium		Silver	*2	Iron	23	Boron	<0.1
Erbium		Palladium		Manganese	1	Beryllium	
Holmium		Rhodium		Chromium	5	Lithium	0.1
Dysprosium		*Heterogeneous				Hydrogen	NR

STD — Internal Standard
NR — Not Reported
All elements not detected < 0.1ppm
MC — Major Component
INT — Interference

Approved M. L. Jacobs by PK Taylor
24 Aug '81

COMMERCIAL TESTING & ENGINEERING CO.
GENERAL OFFICES: 228 NORTH LA SALLE STREET, CHICAGO, ILLINOIS 60601 · AREA CODE 312 726-8434
Reply to INSTRUMENTAL ANALYSIS DIVISION, 14335 WEST 44TH AVENUE, GOLDEN, COLORADO 80401, PHONE 303-278-9521

To: Mr. Roy A. Belletto
Acurex Corporation
485 Clyde Avenue
Mountain View,CA 94942

Date August 21, 1981

Release No. 5
P. O. No.: Subcontract SW59159A

Analyst: J. Oldham

Sample No.: A81-05-030-678
EA- 2 IMP 1

IAD No. 97-G852-116-25

SPARK SOURCE MASS SPECTROGRAPHIC ANALYSIS
CONCENTRATION IN µg/ml

ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.
Uranium		Terbium		Ruthenium		Vanadium	0.006
Thorium		Gadolinium		Molybdenum	0.009	Titanium	0.4
Bismuth		Europium		Niobium	0.009	Scandium	
Lead	0.01	Samarium		Zirconium	0.01	Calcium	4
Thallium		Neodymium	0.009	Yttrium	0.001	Potassium	MC
Mercury	NR	Praseodymium	0.002	Strontium	0.03	Chlorine	0.04
Gold		Cerium	0.005	Rubidium	0.02	Sulfur	>5
Platinum		Lanthanum	0.006	Bromine	≤0.01	Phosphorus	0.1
Iridium		Barium	0.2	Selenium	0.01	Silicon	MC
Osmium		Cesium	≤0.001	Arsenic	NR	Aluminum	>0.8
Rhenium		Iodine	0.002	Germanium		Magnesium	0.9
Tungsten		Tellurium		Gallium	0.006	Sodium	>2
Tantalum		Antimony	NR	Zinc	0.2	Fluorine	≈3
Hafnium		Tin	0.07	Copper	0.05	Oxygen	NR
Lutetium		Indium	STD	Nickel	0.1	Nitrogen	NR
Ytterbium		Cadmium	0.001	Cobalt	≤0.001	Carbon	NR
Thulium		Silver	0.1	Iron	2	Boron	0.002
Erbium		Palladium		Manganese	0.03	Beryllium	
Holmium		Rhodium		Chromium	0.02	Lithium	0.001
Dysprosium						Hydrogen	NR

STD — Internal Standard
NR — Not Reported
All elements not detected < 0.001µg/ml
MC — Major Component > 10µg/ml
INT — Interference

Approved: M. L. Jacobs by
24 Aug 81

COMMERCIAL TESTING & ENGINEERING CO.
GENERAL OFFICES: 228 NORTH LA SALLE STREET, CHICAGO, ILLINOIS 60601 · AREA CODE 312 726-8434
Reply to INSTRUMENTAL ANALYSIS DIVISION, 14335 WEST 44TH AVENUE, GOLDEN, COLORADO 80401, PHONE 303-278-9521

To: Mr. Roy A. Belletto
Acurex Corporation
485 Clyde Avenue
Mountian View, CA 94942

Date August 20, 1981

Analyst J. Oldham

Release No. 5
P. O. No.: Subcontract SW59159A

Sample No. A81-05-030-744
EA-2 flyash

IAD No. 97-G852-116-25

SPARK SOURCE MASS SPECTROGRAPHIC ANALYSIS

CONCENTRATION IN PPM WEIGHT

ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.	ELEMENT	CONC.
Uranium	1	Terbium	1	Ruthenium		Vanadium	25
Thorium	6	Gadolinium	4	Molybdenum	0.7	Titanium	MC
Bismuth		Europium	0.8	Niobium	7	Scandium	0.8
Lead	61	Samarium	6	Zirconium	39	Calcium	MC
Thallium		Neodymium	4	Yttrium	20	Potassium	MC
Mercury	NR	Praseodymium	6	Strontium	860	Chlorine	190
Gold		Cerium	35	Rubidium	290	Sulfur	280
Platinum		Lanthanum	35	Bromine	29	Phosphorus	MC
Iridium		Barium	MC	Selenium	4	Silicon	MC
Osmium		Cesium	0.9	Arsenic	NR	Aluminum	MC
Rhenium		Iodine	3	Germanium	0.8	Magnesium	MC
Tungsten	0.7	Tellurium	0.4	Gallium	11	Sodium	MC
Tantalum		Antimony	NR	Zinc	390	Fluorine	≈100
Hafnium	0.7	Tin	0.2	Copper	45	Oxygen	NR
Lutetium		Indium	STD	Nickel	14	Nitrogen	NR
Ytterbium	0.8	Cadmium	1	Cobalt	3	Carbon	NR
Thulium	0.1	Silver	≤0.2	Iron	MC	Boron	13
Erbium	0.6	Palladium		Manganese	MC	Beryllium	<0.1
Holmium	2	Rhodium		Chromium	38	Lithium	2
Dysprosium	3					Hydrogen	NR

STD — Internal Standard
NR — Not Reported
All elements not detected < 0.1ppm
MC — Major Component
INT — Interference

Approved: M.L. Jacobs by [illegible]
24 Aug '81

5.6 GASEOUS (C_1 to C_6) HYDROCARBONS

5.6 GASEOUS (C_1 to C_6) HYDROCARBONS

Onsite Gas Chromatography Results[a]

Dry Wood Fuel, 4-15-81

Time	Run No.	C_1	C_2	C_3	C_4	C_5	C_6
18:53	16	39.5	43.2	2.8	5.7	4.9	1.3
18:56	17	16.9	18.0	2.9	1.3	0.8	1.9

Wet Wood Fuel, 4-16-81

Time	Run No.	C_1	C_2	C_3	C_4	C_5	C_6
12:45	1	5.2	5.9	1.8	7.1	<3.8[b]	<8.5[b]
13:01	2	4.6	2.0	2.6	--[c]	0.1	<4.6[b]
16:35	3	1.3	4.0	--	0.4	--	<1.5[b]
16:40[d]	4	6.1	3.0	2.0	0.4	0.6	<7.7[b]

[a] All ppm values ±10 percent

[b] Value is higher than actual due to excessive noise

[c] Not detected

[d] Burnout in furnace

GAS CHROMATOGRAPH OPERATING CONDITIONS AND FIELD LOG

Client CMEA, Location ETHAN ALLEN OLD FORT, N.C. Job No. 307602.71

Injection Date 4-15-81, Time 13:16:55, Instrument ID CARLE

Recorder/Printout Reference No. 10, Recorder ID 3390A

Purpose of Run CALIBRATION C_1-C_6 HYDROCARBONS

Sample Description SCOTTY I MULTI-COMPONENT MIXTURE C_1-C_6 n-PARAFINS ±10% BAL N_2

GC CONDITIONS

Amount Injected 2.0ML, Inj. Port or Sample Loop Used 2.0ML LOOP

Detector Used: FID X, ECD __, FPD __, TCD __ (Current ____)

Detector Attenuation 1, Amplifier or Range 10^{-1}

Column: Liquid Phase ____, Solid Phase PORAPAK Q,

Length 6', O.D. 1/8", I.D. ____, Material S.S.

Temperature: Injector 130 °C, Oven 130 °C, Detector 130 °C

Temperature Program ISOTHERMAL

SAMPLE RUN

Sampling Method PURGE LOOP

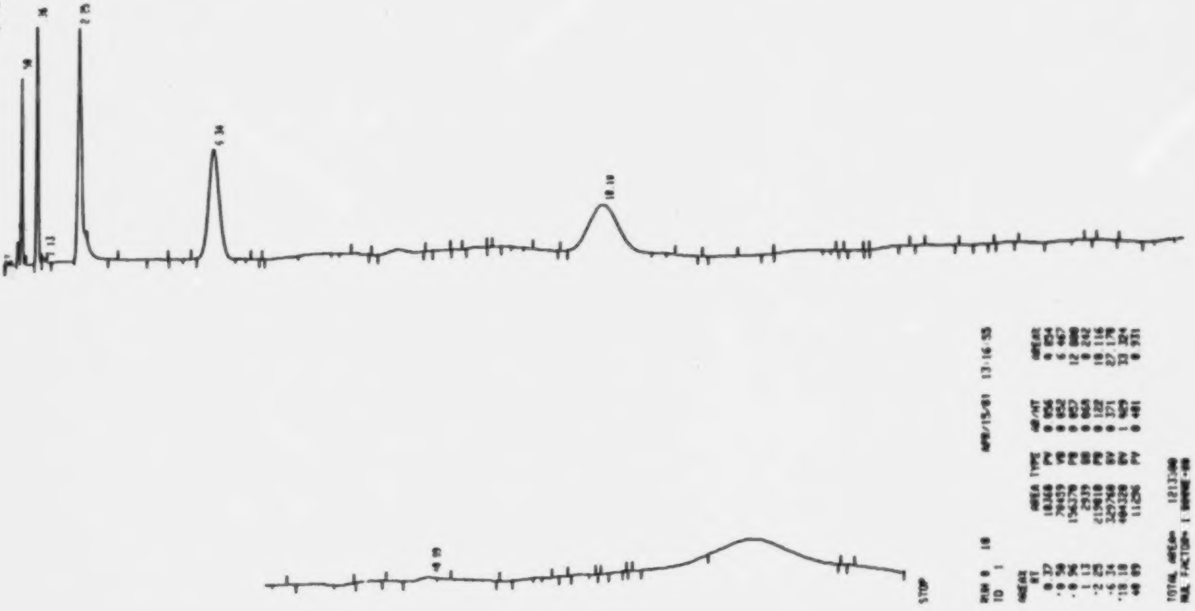
RT	Area	Peak Height	Amount (PPM)	Component
.50	78459		15.1	C_1
.96	156370		14.6	C_2
2.25	219810		15.6	C_3
6.34	329760		15.2	C_4
18.18	404320		15.6	C_5
~48	—		15.9	C_6

Name of Operator M.D. CHIPS, Date 7-8 1981

Operator M. D. CHIPS Date 9-15-81
Column No. Length 6' Dia. 1/8"
Coating Concn.
Support PORAPAK Q Mesh 60/80
TEMP: Col: Init. 130 °C Final 130 °C
Rate °C/min. Det. 130 °C Inj. 130 °C
CARRIER GAS He Rate 18 psi ~~ml/min.~~
Pressures: Inlet Outlet
Hydrogen 30 psi ~~ml/min.~~ Air 60 psi ~~ml/min.~~
DETECTOR E.C. T.C. F.I.D. X
Scavenger Rate ml./min.
Sens. Rec.Range mv.
SAMPLE CALIBRATION 1316 Size 2.0
Solvent Concn.

Carle GC
8801
0801 118°C

Cal C1-C6 Comp
15 ppm ea [illegible]
1-9801



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RUN #   18              APR/15/81   13:16:55
ID   1

AREA%
   RT          AREA TYPE   AR/HT       AREA%
   0.37       10368   PV   0.056       0.854
  *0.58       78459   VB   0.052       6.467
  *0.96      156370   PB   0.057      12.888
   1.13        2939   BB   0.068       0.242
  *2.25      219818   PB   0.122      18.116
  *6.34      329760   BV   0.371      27.178
 *18.18      404320   BV   1.429      33.324
  40.89       11296   PV   0.481       0.931

TOTAL AREA=    1213300
MUL FACTOR= 1.0000E+00
```

GAS CHROMATOGRAPH OPERATING CONDITIONS AND FIELD LOG

Client CMEA, Location ETHAN ALLEN OLD FORT, N.C. Job No. 307602.71

Injection Date 4-15-81, Time 18:42:14, Instrument ID CARLE

Recorder/Printout Reference No. 15, Recorder ID 3390A

Purpose of Run CALIBRATION C_1-C_6 HYDROCARBONS

Sample Description SCOTTY I MULTI-COMPONENT MIXTURE C_1-C_6 n-PARAFINS ±10% bal N_2

GC CONDITIONS

Amount Injected 2.0 ML, Inj. Port or Sample Loop Used 2.0 ML LOOP

Detector Used: FID X, ECD ___, FPD ___, TCD ___ (Current ______)

Detector Attenuation 1, Amplifier or Range 10^{-1}

Column: Liquid Phase ______, Solid Phase PORAPAK Q,

Length 6', O.D. 1/8", I.D. ______, Material S.S.

Temperature: Injector 130 °C, Oven 130 °C, Detector 130 °C

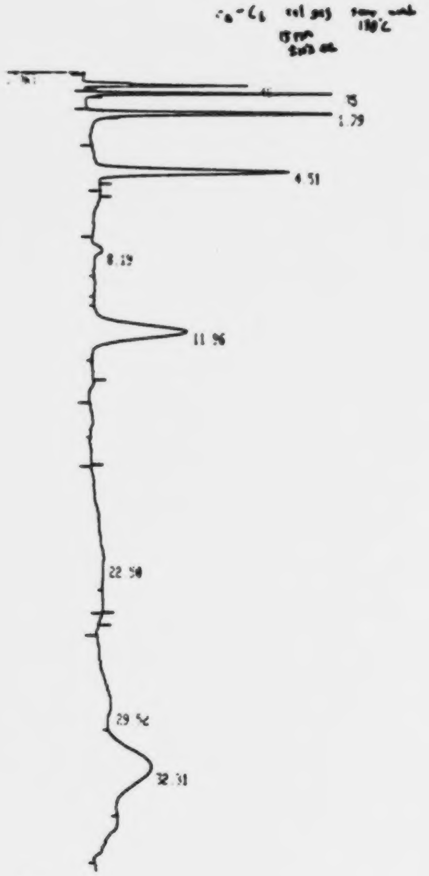
Temperature Program ISOTHERMAL

SAMPLE RUN

Sampling Method PURGE LOOP

RT	Area	Peak Height	Amount (PPM)	Component
.48	100560		15.1	C_1
.85	157470		14.6	C_2
1.79	248840		15.6	C_3
4.51	323360		15.2	C4
11.96	445460		15.6	C5
32.31	803560		15.7	C_6

Name of Operator M.D. CHIPS, Date 7-8 1981



Operator M. D. CHIPS Date 4-15-81
Column No. Length 6' Dia. 1/8"
Coating Concn.
Support PORAPAK Q Mesh 60/80
TEMP: Col: Init. 130 °C Final 130 °C
Rate °C/min. Det 130 °C Inj. 130 °C
CARRIER GAS N2 Rate 60 psi ~~ml/min.~~
Pressures: Inlet Outlet
Hydrogen 40 psi ~~ml/min.~~ Air 60 psi ~~ml/min.~~
DETECTOR E.C. T.C. F.I.D. X
Scavenger Rate ml/min.
Sens. Rec.Range mv.
SAMPLE CALIBRATION 1M2 Size 2.0 ml
Solvent Concn.

```
STOP

RUN 8   15               APR/15/81  18:42:14
ID   1

ESTD
  RT          AREA TYPE  CAL8        AMOUNT
  0.48      100560 D PB    1R        19.353
  0.85      157470 0 PB    2R        14.703
  1.73      248840   PB               0.000
  4.51      323360   BB               0.000
  8.19       36677   PV               0.000
 11.96      445460   VP               0.000
 22.50      102520   PV               0.000
 29.52      218210   BV               0.000
 32.31      803560   VV               0.000

TOTAL AREA=    2436600
MUL FACTOR= 1.0000E+00
```

GAS CHROMATOGRAPH OPERATING CONDITIONS AND FIELD LOG

Client CMEA, Location ETHAN ALLEN OLD FORT, N.C. Job No. 307602.71

Injection Date 4-15-81, Time 17:33:49, Instrument ID CARLE

Recorder/Printout Reference No. 16, Recorder ID 3390A

Purpose of Run C_1-C_6 HYDROCARBON ANALYSIS

Sample Description 1853 ΨBULB

GC CONDITIONS

Amount Injected 2.0ML, Inj. Port or Sample Loop Used 2.0ML LOOP

Detector Used: FID X, ECD ___, FPD ___, TCD ___ (Current ______)

Detector Attenuation 1, Amplifier or Range 10^{-1}

Column: Liquid Phase ______, Solid Phase PORAPAK Q,

Length 6', O.D. 1/8", I.D. ______, Material S.S.

Temperature: Injector 130 °C, Oven 130 °C, Detector 130 °C

Temperature Program ISOTHERMAL

SAMPLE RUN

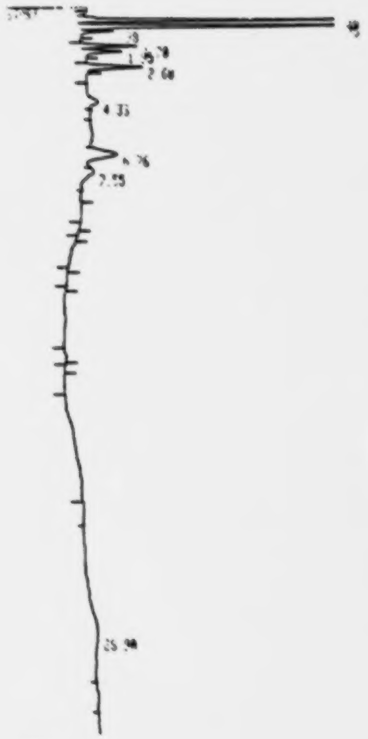
Sampling Method GRAB 300ML

RT	Area	Peak Height	Amount (PPM)	Component
.49	263130		39.5	C_1
.75	466170		43.2	C_2
.98	5347			
1.70	39154		2.8	C_3
1.95	55141			
2.68	54033		5.7	C_4
4.33	32762			

Name of Operator M.D. CHIPS, Date 7-8 1981

RT	Area	Peak Height	Amount (ppm)	Component
6.76	96820		4.9	C_5
7.55	41926			
28.90	66945		1.3	C_6

Sample Bulb Y
taken 1853
130°C



```
STOP


RUN #   16               APR/15/81  19:33:49
ID   1

ESTD
  RT          AREA TYPE  CAL#        AMOUNT
  0.49      263130 D PP   1          50.641
  0.75      466170 D PB   2          43.525
  0.98       5347 D PB                0.000
  1.70       39154   BV   3           2.779
  1.95       35141   VB               0.000
  2.62       54033   PB               0.000
  4.33       32762   BV   4           1.510
  6.76       36820   VV               0.000
  7.55       41926   VP               0.000
 28.94       66945   PV   6           1.326

TOTAL AREA=     1101400
MUL FACTOR= 1.0000E+00
```

Operator. M.D. CHIPS .... Date 4-15-81
Column No. .... Length 6' .... Dia. 1/8"
Coating .... Concn. ....
Support. PORAPAK Q .... Mesh 60/80 ....
TEMP: Col: Init. 130 °C Final 130 °C
Rate .... °C/min. Det. 130 °C Inj. 130 °C
CARRIER GAS He .... Rate 60 PSI ~~ml/min.~~
Pressures: Inlet .... Outlet ....
Hydrogen 40 PSI ~~ml/min.~~ Air 600 PSI ~~ml/min.~~
DETECTOR E.C. .... T.C. .... F.I.D. X
Scavenger .... Rate .... ml/min.
Sens. .... Rec.Range .... mv.
SAMPLE 1853 Y-BULB .... Size 2.0 ml ....
Solvent .... Concn. ....

GAS CHROMATOGRAPH OPERATING CONDITIONS AND FIELD LOG

Client CMEA, Location ETHAN ALLEN OLD FORT, N.C. Job No. 307602.71

Injection Date 4-15-81, Time 20:10:00, Instrument ID CARLE
Recorder/Printout Reference No. 17, Recorder ID 3390A
Purpose of Run C_1-C_6 HYDROCARBON ANALYSIS

Sample Description 1856 # BULB

GC CONDITIONS

Amount Injected 2.0ML, Inj. Port or Sample Loop Used 2.0ML LOOP
Detector Used: FID X, ECD ___, FPD ___, TCD ___ (Current _____)
Detector Attenuation 1, Amplifier or Range 10^{-1}
Column: Liquid Phase _____, Solid Phase PORAPAK Q,
Length 6', O.D. 1/8", I.D. _____, Material S.S.
Temperature: Injector 130 °C, Oven 130 °C, Detector 130 °C
Temperature Program ISOTHERMAL

SAMPLE RUN

Sampling Method GRAB 300 ML

RT	Area	Peak Height	Amount (PPM)	Component
.49	112470		16.9	C_1
.75	193950		18.0	C_2
.97	15066			
1.65	14274		2.9	C_3
1.89	16764			
2.57	26955		1.3	C_4
6.39	23705		0.8	C_5

Name of Operator M.D. CHIPS, Date 7-8 1981

RT	Area	Peak Height	Amount (ppm)	Component
17.67	0	)		)
21.37	96477		1.9	C_6

Sample Inj.

Sample bulb #
taken [illegible]
same cond.
129°C

```
LIST: ZERO =  20,-0.0
LIST: ZERO =  20,-0.2
LIST: ZERO =  20, 0.0
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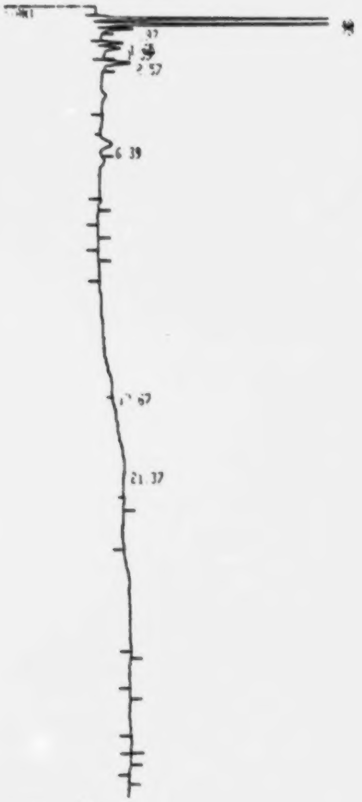


```
STOP


RUN #   17              APR/15/81  20:10:00
ID   1

ESTD
  RT          AREA TYPE  CAL#        AMOUNT
  0.49      112470 D PP    1         21.646
  0.73      193950 D PB    2         18.109
  0.97       15066 D BB               0.000
  1.63       14274   BV               0.000
  1.89       16764   VB    3          1.190
  2.57       26955   BB               0.000
  6.39       23705   PB               0.000
 17.67           0   PV               0.000
 21.37       96477   VV               0.000

TOTAL AREA=     493660
MUL FACTOR= 1.0000E+00
```

Operator M.D. CHIPS Date 4-15-81
Column No. Length 6' Dia. 1/8"
Coating Concn.
Support PORAPAK Q Mesh 60/80
TEMP: Col: Init. 130 °C Final 130 °C
Rate °C/min. Det. 130 °C Inj. 130 °C
CARRIER GAS He Rate 60 psi ~~ml/min.~~
Pressures: Inlet Outlet
Hydrogen 40 psi ~~ml/min~~ Air 60 psi ~~ml/min.~~
DETECTOR E.C. T.C. F.I.D. X
Scavenger Rate ml/min.
Sens. Rec.Range mv.
SAMPLE 1856 → Size 2.0 ml.
Solvent Concn.

GAS CHROMATOGRAPH OPERATING CONDITIONS AND FIELD LOG

Client CMEA, Location ETHAN ALLEN OLD FORT, N.C. Job No. 307602.71

Injection Date 4-16-81, Time 11:04:46, Instrument ID CARLE

Recorder/Printout Reference No. 21, Recorder ID 3390A

Purpose of Run CALIBRATION C_1-C_6 HYDROCARBONS

Sample Description SCOTTY I MULTICOMPONENT MIXTURE C_1-C_6 n-PARAFINS ±10% Bal N_2

GC CONDITIONS

Amount Injected 2.0 ML, Inj. Port or Sample Loop Used 2.0 ML LOOP

Detector Used: FID X, ECD ___, FPD ___, TCD ___ (Current ______)

Detector Attenuation 1, Amplifier or Range 10^{-1}

Column: Liquid Phase ______, Solid Phase PORAPAK Q,

Length 6', O.D. 1/8", I.D. ______, Material S.S.

Temperature: Injector 130 °C, Oven 130 °C, Detector 130 °C

Temperature Program ISOTHERMAL

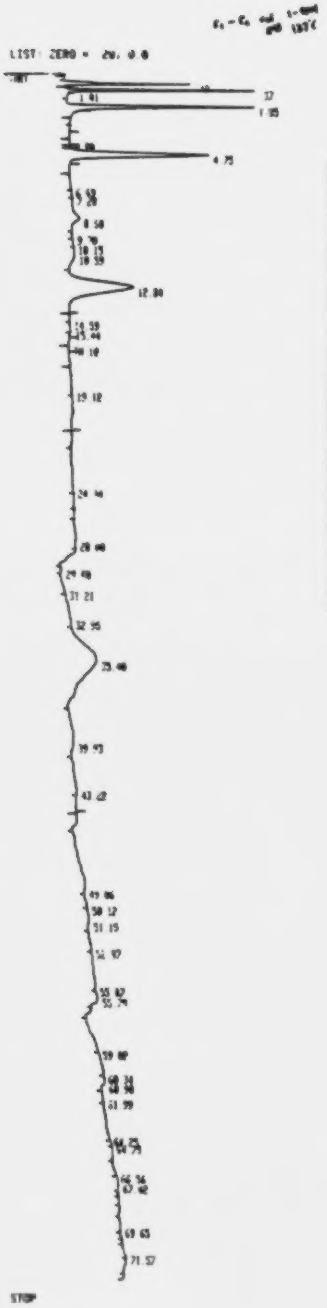
SAMPLE RUN

Sampling Method PURGE LOOP

RT	Area	Peak Height	Amount (PPM)	Component
.49	95557		15.1	C_1
.87	155030		14.6	C_2
1.85	238150		15.6	C_3
4.75	318790		15.2	C_4
12.84	394380		15.6	C_5
35.40	685190		15.9	C_6

Name of Operator M.D. CHIPS, Date 7-8 1981

Operator M.D. CHIPS Date 4-16-81
Column No. Length 6' Dia. 1/8"
Coating Concn.
Support PIRAPAK Q Mesh 60/80
TEMP: Col: Init 130 °C Final 130 °C
Rate °C/min. Det. 130 °C Inj. 130 °C
CARRIER GAS He Rate 18 psi ~~ml./min.~~
Pressures: Inlet Outlet
Hydrogen 40 psi ~~ml./min.~~ Air 60 psi ~~ml./min.~~
DETECTOR E.C. T.C. F.I.D. X
Scavenger Rate ml./min.
Sens. Rec.Range mv.
SAMPLE CALIBRATION 1104 Size 2.0 ML
Solvent Concn.



```
RUN #   21                APR/16/81  11:04:46
ID   1

ESTD
   RT         AREA TYPE  CAL#       AMOUNT
  0.49       95557 D VB   1         10.391
  0.82      155030 D PV   2         14.475
  1.81        8423 D VP              0.000
  1.95      238150   PB   3         16.982
  4.80         611   PB              0.000
  4.75      318790   BB   4         14.695
  6.69        7491   BV              0.000
  7.28       11285   VV              0.000
  8.60       76317   VV              0.000
  9.70       12711   VV              0.000
 10.15       15951   VV              0.000
 10.59       42604   VP              0.000
 12.84      394300   PB   5         15.217
 14.59        2070   BV              0.000
 15.44        4799   VV              0.000
 19.12       19709   PV              0.000
 24.94      100310   VV              0.000
 29.80      169040   VV              0.000
 29.80        3333   PV              0.000
 31.21       17765   VV              0.000
 32.95       93625   VV              0.000
 35.40      685190   VP   6         13.567
 39.93       51054   PP              0.000
 43.22       29206   PV              0.000
 49.06      161940   PV              0.000
 50.12       59530   VV              0.000

ESCAPE
```

GAS CHROMATOGRAPH OPERATING CONDITIONS AND FIELD LOG

Client CMEA, Location ETHAN ALLEN OLD FORT, N.C. Job No. 307602.71

Injection Date 4-16-81, Time 15:09:47, Instrument ID CARLE
Recorder/Printout Reference No. 1, Recorder ID 3390A
Purpose of Run C_1-C_6 HYDROCARBON ANALYSIS

Sample Description 1245 4 BULB

GC CONDITIONS

Amount Injected 2.0ML, Inj. Port or Sample Loop Used 2.0ML LOOP
Detector Used: FID X, ECD ___, FPD ___, TCD ___ (Current ________)
Detector Attenuation 1, Amplifier or Range 10^{-1}
Column: Liquid Phase ____________, Solid Phase PORAPAK Q,
Length 6', O.D. 1/8", I.D. ________, Material S.S.
Temperature: Injector 130 °C, Oven 130 °C, Detector 130 °C
Temperature Program ISOTHERMAL

SAMPLE RUN

Sampling Method 300ML GRAB

RT	Area	Peak Height	Amount (PPM)	Component
.50	26970		5.2	C_1
.79	6326		5.9	C_2
.91	731	)		)
1.03	22793		1.8	C_3
1.86	1868			
2.04	292	)		)
2.19	1409			

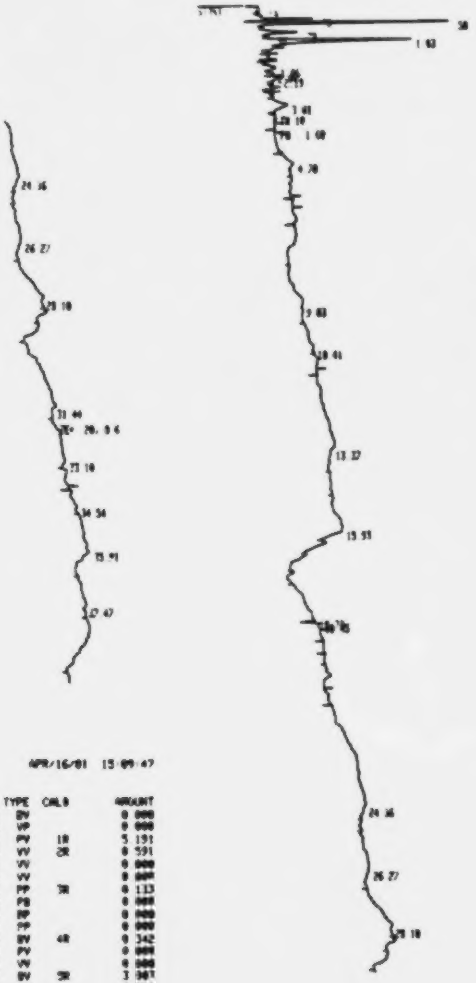
↑ OVER

Name of Operator M.D. CHIPS, Date 7-8 1981

RT	Area	Peak Height	Amount (ppm)	Component
3.01	6380			C_4
4.78	7427		7.1	
9.03	0			
10.41	316		< 3.8	C_5
13.37	98567			
15.93	123980			
18.72	19957			
18.85	2038			
24.36	93925			
26.27	36193		< 8.5	C_6
28.10	41433			
31.44	32466			
33.10	18626			
34.54	6446			
35.91	51773			

```
LIST: ZERO = 20.0 0
```

LT Bulb sample Y
[illegible] 128°F
manifold 128°C
[illegible]



Operator M.D. Wiles Date 4-16-81
Column No. Length 6' Dia. 1/8"
Coating Conc.
Support PORAPAK Q Mesh 60/80
TEMP: Col: Init. 130 °C Final 130 °C
Rate °C/min. Det. 130 °C Inj. 130 °C
CARRIER GAS He Rate 18 psi ~~ml/min.~~
Pressures: Inlet Outlet
Hydrogen 40 psi ~~ml/min.~~ Air 60 psi ml/min.
DETECTOR E.C. T.C. F.I.D. X
Scavenger Rate ml/min.
Sens. Rec.Range mv.
SAMPLE 1245 Y Bulb Size 2.0 mL
Solvent Conc.

```
STOP

RUN 0     1              APR/16/81   15:09:47

ESTD
  RT           AREA TYPE  CAL#        AMOUNT
  0.10         1394  BV                0.000
  0.33         4085  VP                0.000
  0.50        26970  PV     1R         5.191
  0.73         6326  VV     2R         0.591
  0.91          731  VV                0.000
  1.03        22793  VV                0.000
  1.06         1068  PP     3R         0.113
  2.04          292  PB                0.000
  2.19         1409  BP                0.000
  3.01         6380  PP                0.000
  4.78         7427  BV     4R         0.342
  9.03            9  PV                0.000
 10.41          316  VV                0.000
 13.37        90567  BV     5R         3.387
 15.93       123980  VV                0.000
 18.72        19957  PP                0.000
 18.85         2030  PB                0.000
 24.36        93925  BV                0.000
 26.27        36193  VV                0.000
 29.10        41433  VV                0.000
 31.44        32466  PV                0.000
 33.10        13626  VP                0.000
 34.54         6446  BV                0.000
 35.91        51773  VV     6R         1.823
 37.47        31530  VV                0.000

TOTAL AREA=    641720
MUL FACTOR= 1.0000E+00
```

GAS CHROMATOGRAPH OPERATING CONDITIONS AND FIELD LOG

Client CMEA, Location ETHAN ALLEN Job No. 307602.71
OLD FORT, N.C.

Injection Date 4-16-81, Time 15:55:05, Instrument ID CARLE

Recorder/Printout Reference No. 2, Recorder ID 3390A

Purpose of Run C_1-C_6 HYDROCARBON ANALYSIS

Sample Description 1301 [illegible] BULB

GC CONDITIONS

Amount Injected 2.0 ML, Inj. Port or Sample Loop Used 2.0 ML LOOP

Detector Used: FID X, ECD __, FPD __, TCD __ (Current ____)

Detector Attenuation 1, Amplifier or Range 10^{-1}

Column: Liquid Phase ____, Solid Phase PORAPAK Q,

Length 6', O.D. 1/8", I.D. ____, Material S.S.

Temperature: Injector 130 °C, Oven 130 °C, Detector 130 °C

Temperature Program ISOTHERMAL

SAMPLE RUN

Sampling Method 300 ML GRAB

RT	Area	Peak Height	Amount (PPM)	Component
.49	24063		4.6	C_1
.75	21089		2.0	C_2
.97	32413		2.6	C_3
14.07	2946		0.1	C_5
20.02	10699			C_6
25.52	21895			
30.35	12399			

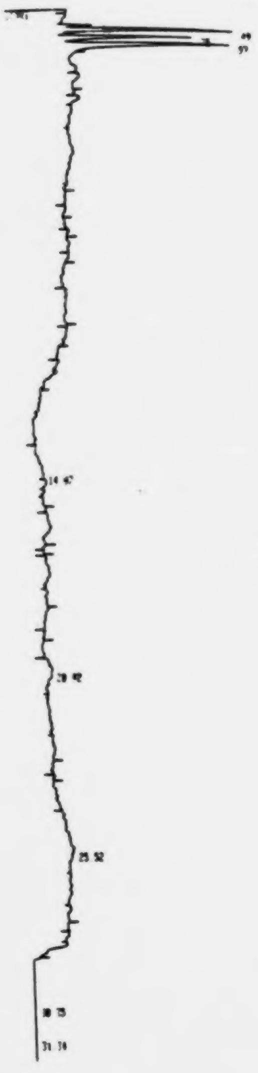
↓ OVER

Name of Operator M.D. CHIPS, Date 7-8 1981

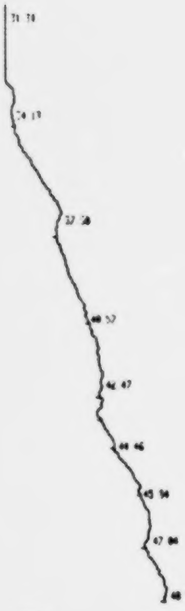
RT	Area	Peak Height	Amount (ppm)	Component
31.34	34590			C_6
34.13	43132		<4.6	
37.50	107490			

```
LIST  ZERO =  20. 0 2
LIST  ZERO =  20. 0 2
LIST  ZERO =  20. 0 1
LIST  ZERO =  20. 0 0
```

But Sample Bulb A
taken 1301
wet and dual
130°C



Operator M.D. CHIPS Date 4-16-81
Column No. Length 6' Dia. 1/8"
Coating Concn.
Support PORAPAK Q Mesh 60/80
TEMP: Col: Init. 130 °C Final 130 °C
Rate °C/min. Det. 130 °C Inj. 130 °C
CARRIER GAS He Rate 18 psi ml/min.
Pressures: Inlet Outlet
Hydrogen 40 psi ml/min. Air 60 psi ml/min.
DETECTOR E.C. T.C. F.I.D. x
Scavenger Rate ml/min.
Sens. Rec.Range mv.
SAMPLE 1301 - BULB Size 2.0 ml.
Solvent Concn.



```
STOP


RUN 0    2                    APR/16/81   15:55 05

ESTD
  RT              AREA TYPE  CAL 0        AMOUNT
  0 38            3660 0 PV               0 000
  0 49           24063 0 VB    1R         4 631
  0 75           21089 0 BP    2R         1 969
  0 97           72413 0 PB               0 000
 14 07            2946   BV    5R         0 114
 20 02           10699   BV               0 000
 25 52           21895   BV               0 000
 30 35           12399   BV               0 000
 31 34           14590   VV               0 000
 34 13           43132   PV               0 000
 37 58          187490   VV    6R         2 129
 40 57           90691   VV               0 000
 42 47           36640   VV               0 000
 44 46            5354   PV               0 000
 45 84           31081   VV               0 000
 47 94           41135   VP               0 000
 48 96           11924   PP               0 000

TOTAL AREA=     581118
MUL FACTOR= 1 0000E+00
```

GAS CHROMATOGRAPH OPERATING CONDITIONS AND FIELD LOG

Client CMEA, Location ETHAN ALLEN Job No. 307602.71
OLD FORT, N.C.

Injection Date 4-16-81, Time 16:46:35, Instrument ID CARLE
Recorder/Printout Reference No. 3, Recorder ID 3390A
Purpose of Run C_1-C_6 HYDROCARBON ANALYSIS
Sample Description 1635 ψ BULB

GC CONDITIONS

Amount Injected 2.0ML, Inj. Port or Sample Loop Used 2.0ML LOOP
Detector Used: FID X, ECD __, FPD __, TCD __ (Current ______)
Detector Attenuation 1, Amplifier or Range 10^{-1}
Column: Liquid Phase ______, Solid Phase PORAPAK Q,
Length 6', O.D. 1/8", I.D. ______, Material S.S.
Temperature: Injector 130 °C, Oven 130 °C, Detector 130 °C
Temperature Program ISOTHERMAL

SAMPLE RUN

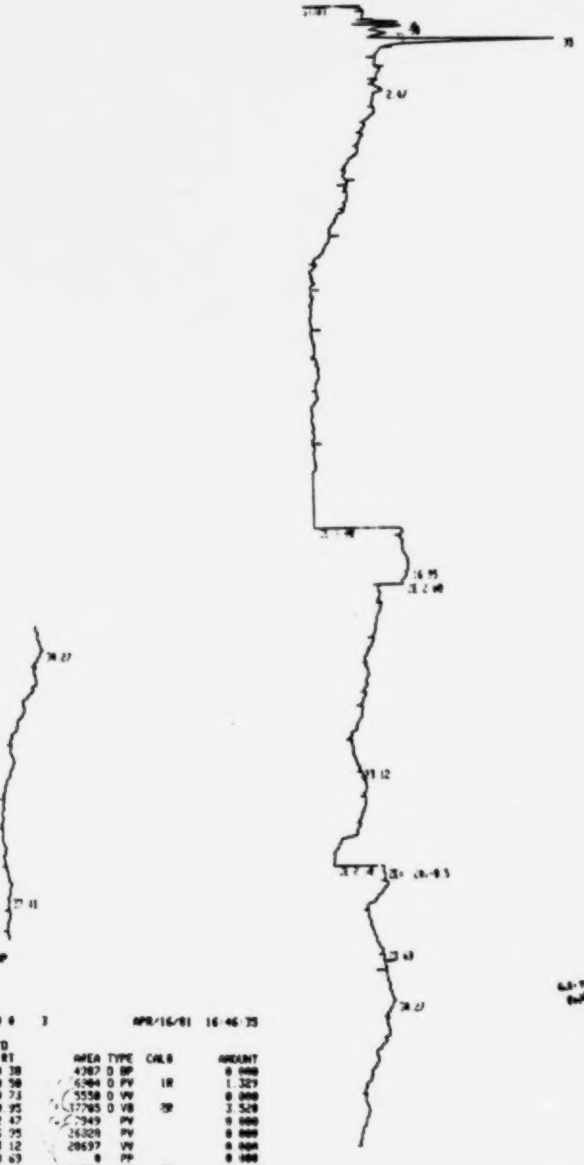
Sampling Method 300ML GRAB

RT	Area	Peak Height	Amount (PPM)	Component
.50	6904		1.3	C_1
.73	5550		4.0	C_2
.95	37705			
2.47	7949		0.4	C4
16.95	26828		<1.5	C_6
23.12	28697			
28.69	0			
30.27	21869			

Name of Operator M.D. CHIPS, Date 7-8 1981

```
LIST: ZERO =  20. 0.5
LIST: ZERO =  20.-0.2
LIST: ZERO =  20. 0.0
```

Hot body?
sample tubing
1635
138°C



```
STOP

RUN #     3                  APR/16/81  16:46:35

ESTD
   RT            AREA TYPE  CAL#          AMOUNT
   0.38          4387 D BP                0.000
   0.50          6204 D PV    1R          1.329
   0.73          5550 D VV                0.000
   0.95         37705 D VB    3R          3.520
   2.47          7949   PV                0.000
  16.95         26329   PV                0.000
  23.12         20637   VV                0.000
  29.63             0   PP                0.000
  30.27         21069   BV    6R          0.433
  37.41          9171   PV                0.000

TOTAL AREA=      149660
MUL FACTOR= 1.0000E+00
```

Operator M.D. CHIPS Date 4-16-81
Column No. Length 6' Dia. 1/8"
Coating Concn.
Support PORAPAK Q Mesh 60/80
TEMP: Col: Init. 130 °C Final 130 °C
Rate °C/min. Det. 130 °C Inj. 170 °C
CARRIER GAS He Rate 18 psi ~~ml/min.~~
Pressures: Inlet Outlet
Hydrogen 30 psi ~~ml/min.~~ Air 60 psi ~~ml/min.~~
DETECTOR E.C. T.C. F.I.D. X
Scavenger Rate ml/min.
Sens. Rec.Range mv.
SAMPLE 1635 4-80-6 Size 2.0ML
Solvent Concn.

GAS CHROMATOGRAPH OPERATING CONDITIONS AND FIELD LOG

Client CMEA, Location ETHAN ALLEN OLD FORT, N.C. Job No. 307602.71

Injection Date 4-16-81, Time 17:26:59, Instrument ID CARLE

Recorder/Printout Reference No. 4, Recorder ID 3390A

Purpose of Run C_1-C_6 HYDROCARBON ANALYSIS

Sample Description 1140 # BULB

GC CONDITIONS

Amount Injected 2.0ML, Inj. Port or Sample Loop Used 2.0ML LOOP

Detector Used: FID X, ECD __, FPD __, TCD __ (Current ____)

Detector Attenuation 1, Amplifier or Range 10^{-1}

Column: Liquid Phase ____, Solid Phase PORAPAK Q,

Length 6', O.D. 1/8", I.D. ____, Material S.S.

Temperature: Injector 130 °C, Oven 130 °C, Detector 130 °C

Temperature Program ISOTHERMAL

SAMPLE RUN

Sampling Method 300ML GRAB

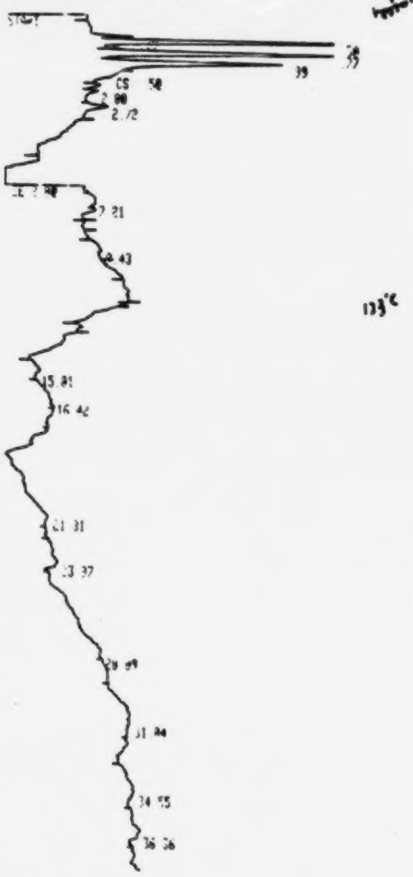
RT	Area	Peak Height	Amount (PPM)	Component
.50	31803		6.1	C_1
.77	32259		3.0	C_2
.99	26171			C_3
2.00	2818		2.0	
2.72	9199		0.4	C_4
7.21	10927			C_5
9.43	4220		0.6	

Name of Operator M.D. CHIPS, Date 7-8 1981

RT	Area	Peak Height	Amount (ppm)	Component
15.01	13011			
16.42	46608			
21.81	50532		< 7.7	C_6
23.97	36778			
28.09	109150			
31.04	132990			

```
LIST: ZERO =  20,-0.7
LIST: ZERO =  20,-0.2
LIST: ZERO =  20,-0.1
LIST: ZERO =  20, 0.0
```

S-J. Hot Bulb
Sample ∝
Teflon 1640
186°C
Boiler [illegible]



```
STOP

RUN #    4              APR/16/81  17:26:59

ESTD
  RT          AREA TYPE  CAL#        AMOUNT
  0.39        2454 D PV               0.000
  0.50       31803 D VB   1R          6.121
  0.77       32259 D BP   2R          3.012
  0.99       26171 D PA               0.000
  2.00        2318   PP   3R          0.200
  2.72        9199   PB               0.000
  7.21       10927   PV               0.000
  9.43        4220   PB               0.000
 15.01       13011   BV               0.000
 15.42       46603   VV               0.000
 21.81       50532   PV               0.000
 23.97       36778   VV               0.000
 28.39      109150   VV               0.000
 31.04      132990   VV   6R          2.633
 34.55       61310   VV               0.000
 36.35       29360   VP               0.000

TOTAL AREA=     599590
MUL FACTOR= 1.0000E+00
```

Operator M.D. CHIPS Date 4-16-81
Column No. Length 6' Dia. 1/8"
Coating Concn.
Support PORAPAK Q Mesh
TEMP: Col: Init 130 °C Final 130 °C
Rate °C/min. Det. 130 °C Inj. 130 °C
CARRIER GAS He Rate 18 psi ~~ml/min.~~
Pressures: Inlet Outlet
Hydrogen 40 psi ~~ml/min.~~ Air 60 psi ~~ml/min.~~
DETECTOR E.C. T.C. F.I.D. X
Scavenger Rate ml./min.
Sens. Rec.Range mv.
SAMPLE 1640 ∝ Size 2.0 mL
Solvent Concn.

5.7 TOTAL CHROMATOGRAPHABLE (TCO) AND GRAVIMETRIC ORGANICS, INFRARED SPECTRA (IR), AND GAS CHROMATOGRAPHY/MASS SPECTROMETRY (GC/MS) OF TOTAL SAMPLE EXTRACTS

ACUREX
Corporation

Energy & Environmental Division

Acurex
M.S. 2-2260

August 3, 1981
Acurex ID #A81-05-030
A81-05-031
Client P.O. #307736.12

Attention: L. Waterland

Sample: 2 SASS Train, received 5/11/81
1 SASS Train, received 5/29/81

The above referenced samples were analyzed per Level 1 protocol. Arsenic and antimony were determined by furnace AAS.

Polynuclears were determined by a modified EPA method 625. 1 ul of sample was injected onto a SE-54, J and W 30 meter capillary column using Grob injection. The column was held at 30°C. No polynuclears were detected in the GC/MS blanks above 1 ng.

The XAD-2 resin samples and XAD-2 resin blank contained about 120 mg TCO of material that appears to be a product of acetone reacting with XAD-2 resin. Through the use of the TCO and GC/MS chromatograms, the blank TCO runs were subtracted to give the data in the report.

Benzo (c) phenanthrene, dibenzo (c,g) carbozole, 7, 12-dimethylbenz (a) anthracene, 3-methyl chloranthene, and perylene were not detected (<40 ng/ul injection) in any sample analyzed by GC/MS.

Prepared by: Greg Nicoll
Greg Nicoll
Program Director

Authorized by: Linda Bohannon
Linda K. Bohannon
Sample Control Manager

ACUREX Corporation
ANALYSIS LABORATORIES

DATA REPORTING FORM

Acurex E/S (CMEA)
A81-05-030
Page 2 of 5

CUSTOMER CMEA
DATE October 25, 1982
CUSTOMER CONTRACT NO. 307736.12
ACUREX CONTRACT NO. A81-05-030
RESULTS REPORT TO L. Waterland
TELEPHONE
ADDRESS
Ethan Allen-1 21.62 dscm

SAMPLE ID (CUSTOMER)		10µ + 3µ	1µ+Filter	XAD	OMC	Imp I	Imp 2+3	Fly Ash	Fuel	
SAMPLE ID (LAB)		646	644	650	652	653	655	662	661	
PARAMETER										UNITS
GRAV Aliquot		<4	<4	181	8	--	--	14	--	mg
GRAV (Blank)		<4	<4	<4	<4	--	--	<4	--	mg
GRAV		<0.2	<0.3	9.1	0.4	--	--	700mg/kg	--	mg/dscm
TCO Aliquot		--	--	(250)130*	4.9	--	--	0.4	--	mg
TCO (Blank)		--	--	(120)0.64*	<0.1	--	--	<0.1	--	mg
TCO		--	--	6.0*	0.23	--	--	20mg/kg	--	mg/dscm
Mercury Aliquot		<1	<1	2	--	7	<1	<1	<1	µg/L
Mercury (Blank)		<1	<1	<1	--	<1	<1	<1	<1	µg/L
Mercury		<0.00007	<0.00007	0.0006	--	0.0005	<0.00006	<0.05mg/kg	<0.05mg/kg	mg/dscm
Antimony Aliquot		--	--	--	--	--	<10	--	--	µg/L
Antimony		--	--	--	--	--	<0.0006	--	--	mg/dscm
Arsenic Aliquot		--	--	--	--	--	<10	--	--	µg/L
Arsenic		--	--	--	--	--	<0.0006	--	--	mg/dscm

*Corrected for resin contamination -- uncorrected number in parentheses

ANALYST

REVIEWER

IR REPORT

SAMPLE: 642 EA I Filter Blank

Wave Number (cm^{-1})	Intensity	Assignment	Comments
		No Peaks	

IR REPORT

SAMPLE: 651 EA I XAD Blank

Wave Number (cm^{-1})	Intensity	Assignment	Comments
		No Peaks	

IR REPORT

SAMPLE: 646 EA I 10u & 3u

Wave Number (cm^{-1})	Intensity	Assignment Comments
		No Peaks

IR REPORT

SAMPLE: 644 EA I lu & Filter

Wave Number (cm^{-1})	Intensity	Assignment	Comments
		No Peaks	

IR REPORT

SAMPLE: 650 EA I XAD

Wave Number (cm^{-1})	Intensity	Assignment	Comments
3600-3000	S	O-H COOH	
2900	S	C-H Alkane	
2820	S	C-H Alkane	
1790	S	C=O COOH	
1600	W	C-H Alkane	
1440	M	C-H Alkane	
1180	M	Not assigned	

IR REPORT

SAMPLE: 652 EA I OMC

Wave Number (cm^{-1})	Intensity	Assignment	Comments
		No Peaks	

IR REPORT

SAMPLE: 662 EA I Flyash

Wave Number (cm^{-1})	Intensity	Assignment	Comments
2900	S	C-H Alkane	
2820	S	C-H Alkane	

ACUREX Corporation
ANALYSIS LABORATORIES

DATA REPORTING FORM

CUSTOMER CMEA
DATE July 31, 1981
CUSTOMER CONTRACT NO. 307736.12
ACUREX CONTRACT NO. A31-05-030
RESULTS REPORT TO L. Waterland
TELEPHONE
ADDRESS
Ethan Allen - 1 21.62

SAMPLE ID (CUSTOMER)	10u+3u	1u+Filter	XAD	OMC	Fly Ash					
SAMPLE ID (LAB)	646	644	650	652	662					
PARAMETER										UNITS
Phenol Aliquot	< 1	< 1	94	< 1	< 1					ng
Naphthalene Aliquot	< 1	< 1	90	< 1	< 1					ng
Acenaphthalene Aliquot	< 1	< 1	10	< 1	< 1					ng
Phenanthrene Aliquot	< 1	< 1	140	< 1	< 1					ng
Pyrene Aliquot	< 1	< 1	5	< 1	< 1					ng
Fluorene Aliquot	< 1	< 1	13	< 1	< 1					ng
Benzo/J+K/Fluoranthenes Aliquot	< 1	< 1	2	< 1	< 1					ng

ANALYST
REVIEWER

Form EED-057 4 80

ACUREX Corporation
ANALYSIS LABORATORIES

DATA REPORTING FORM

CUSTOMER CMEA
DATE July 31, 1981
CUSTOMER CONTRACT NO. 307736.12
ACUREX CONTRACT NO. A81-05-030
RESULTS REPORT TO L. Waterland
TELEPHONE
ADDRESS
Ethan Allen - 1 21.62 dscm

SAMPLE ID (CUSTOMER)	10u+3u	1u+Filter	XAD	OMC	Fly Ash					
SAMPLE ID (LAB)	646	644	650	652	662					
PARAMETER										UNITS
Phenol	< 90	< 80	4700	< 50	<0.05mg/kg					ng/dscm
Naphthalene	< 90	< 80	4500	< 50	<0.05mg/kg					ng/dscm
Acenapthalene	< 90	< 80	500	< 50	<0.05mg/kg					ng/dscm
Phenanthrene	< 90	< 80	7000	< 50	<0.05mg/kg					ng/dscm
Pyrene	< 90	< 80	300	< 50	<0.05mg/kg					ng/dscm
Fluorene	< 90	< 80	650	< 50	<0.05mg/kg					ng/dscm
Benxo/J+K/Fluoranthenes	< 90	< 80	100	< 50	<0.05mg/kg					ng/dscm
Others with a detection limit of 1 ng	< 90	< 80	< 50	< 50	<0.05mg/kg					ng/dscm

ANALYST

REVIEWER

Form EED-057 4 80

ACUREX Corporation
ANALYSIS LABORATORIES

DATA REPORTING FORM

Acurex E/S (CMEA)
A81-05-030
Page 3 of 5

CUSTOMER CMEA
DATE October 25, 1982
CUSTOMER CONTRACT NO. 307736.12
ACUREX CONTRACT NO. A81-05-030
RESULTS REPORT TO L. Waterland
TELEPHONE
ADDRESS
Ethan Allen-2 27.06 dscm

SAMPLE ID (CUSTOMER)		10µ+3µ	1µ + Filter	XAD	OMC	Imp 1	Imp 2+3	Fly Ash	Fuel	
SAMPLE ID (LAB)		672	674	676	677	678	679	744	743	
PARAMETER	▨	▨	▨	▨	▨	▨	▨	▨	▨	UNITS
GRAV Aliquot		<4	<4	35	<4	--	--	13	--	mg
GRAV (Blank)		<4	<4	<4	<4	--	--	<4	--	mg
GRAV		<0.3	<0.3	1.4	<0.1	--	--	650mg/kg	--	mg/dscm
TCO Aliquot		--	--	(140) 20*	0.2	--	--	0.3	--	mg
TCO (Blank)		--	--	(120)0.64*	<0.1	--	--	<0.1	--	mg
TCO		--	--	0.72*	0.007	--	--	20mg/kg	--	mg/dscm
Mercury Aliquot		<1	<1	<1	--	<1	<1	<1	<1	µg/L
Mercury (Blank)		<1	<1	<1	--	<1	<1	<1	<1	µg/L
Mercury		<0.0003	<0.00006	<0.0002	--	<0.00007	<0.00005	<0.05mg/kg	<0.05mg/kg	mg/dscm
Antimony Aliquot		--	--	--	--	--	<10	--	--	µg/L
Antimony		--	--	--	--	--	<0.0005	--	--	mg/dscm
Arsenic Aliquot		--	--	--	--	--	<10	--	--	µg/L
Arsenic		--	--	--	--	--	<0.0005	--	--	mg/dscm

*Corrected for resin contamination -- uncorrected number in parentheses

ANALYST

REVIEWER

Form EED-057 4/80

IR REPORT

SAMPLE: 672 EA II 10u & 3u

Wave Number (cm^{-1})	Intensity	Assignment Comments
		No Peaks

IR REPORT

SAMPLE: 674 EA II lu & Filter

Wave Number (cm^{-1})	Intensity	Assignment	Comments
2900	S	C-H Alkane	

IR REPORT

SAMPLE: 676 EA II XAD

Wave Number (cm^{-1})	Intensity	Assignment	Comments
2900	S	C-H Alkane	
2820	S	C-H Alkane	
1710	M	C=O	

IR REPORT

SAMPLE: 677 OMC EA II

Wave Number (cm⁻¹)	Intensity	Assignment	Comments
3680-3200	S	O-H	
3100-2700	W	C-H Alkane	

IR REPORT

SAMPLE: 744 EA II Flyash

Wave Number (cm^{-1})	Intensity	Assignment	Comments
2900	S	C-H Alkane	
2820	S	C-H Alkane	

ACUREX Corporation
ANALYSIS LABORATORIES

DATA REPORTING FORM

CUSTOMER CMEA
DATE July 31, 1981
CUSTOMER CONTRACT NO. 307736.12
ACUREX CONTRACT NO. A81-05-030
RESULTS REPORT TO L. Waterland
TELEPHONE
ADDRESS
Ethan Allen - 2 27.06 dscm

SAMPLE ID (CUSTOMER)	10u+3u	1u+Filter	XAD	OMC	Fly Ash					
SAMPLE ID (LAB)	672	674	676	677	744					
PARAMETER										UNITS
Acenaphthylene Aliquot	< 1	< 1	130	< 1	< 1					ng
Acenaphthene Aliquot	< 1	< 1	3	< 1	< 1					ng
Phenanthrene Aliquot	< 1	< 1	49	< 1	< 1					ng
Anthracene Aliquot	< 1	< 1	5	< 1	< 1					ng
Fluoranthene Aliquot	< 1	< 1	7	< 1	< 1					ng
Pyrene Aliquot	< 1	< 1	5	< 1	< 1					ng
Chrysene Aliquot	< 1	< 1	1	< 1	< 1					ng
Phenol Aliquot	< 1	< 1	< 1	26	< 1					ng

ANALYST
REVIEWER

ACUREX Corporation
ANALYSIS LABORATORIES

DATA REPORTING FORM

CUSTOMER CMEA

DATE July 31, 1981

CUSTOMER CONTRACT NO. 307736.12

ACUREX CONTRACT NO. A81-05-030

RESULTS REPORT TO L. WAterland

TELEPHONE

ADDRESS

Ethan Allen - 2 27.06 dscm

SAMPLE ID (CUSTOMER)	10u+3u	1u+Filter	XAD	OMC	Fly Ash					
SAMPLE ID (LAB)	672	674	676	677	744					
PARAMETER										UNITS
Acenaphthylene	< 50	< 50	5200	< 40	< 0.05mg/kg					ng/dscm
Acenaphthene	< 50	< 50	100	< 40	< 0.05mg/kg					ng/dscm
Phenanthrene	< 50	< 50	2000	< 40	< 0.05mg/kg					ng/dscm
Anthracene	< 50	< 50	200	< 40	< 0.05mg/kg					ng/dscm
Fluoranthene	< 50	< 50	300	< 40	< 0.05mg/kg					ng/dscm
Pyrene	< 50	< 50	200	< 40	< 0.05mg/kg					ng/dscm
Chrysene	< 50	< 50	40	< 40	< 0.05mg/kg					ng/dscm
Phenol	< 50	< 50	< 40	960	< 0.05mg/kg					ng/dscm
Others with a detection limit of 1 ng	< 50	< 50	< 40	< 40	< 0.05mg/kg					ng/dscm

ANALYST

REVIEWER

Form EED-057 4 80

5.8 LIQUID CHROMATOGRAPHY (LC) SEPARATION AND INFRARED SPECTRA OF LC FRACTIONS

ACUREX Corporation

Energy & Environmental Division

CMEA/Acurex

October 5, 1981
Lab ID Number: A81-08-023
Customer P.O. Number: 307736.12

ATTENTION: L. Waterland

Samples: Ethan Allen XAD extracts (3)

The above referenced samples from earlier work were analyzed by Level 1 procedures. The TCO, GRAV and IR results from the LC fractions are included.

Prepared by: Viorica Lopez-Avila, Ph.D.
GC/MS Group Leader

Approved by: Greg Nicoll
Greg Nicoll
Program Director

VLA/GN:es

485 CLYDE AVENUE. MOUNTAIN VIEW, CA 94042 PHONE (415) 964-3200 TELEX 34-6391 TWX 910-3736593

SAMPLE: EAI XAD #650

	TCO mg	GRAV mg	TCO + GRAV Total mg	Concentration mg/dscm
Total Sample[1]	130	196	326	15
Taken for LC[2]	47	71	118	5.5
Recovered[3]	16	50	66	3.1

Fraction	TCO in mg				GRAV in mg				TCO + GRAV Total mg	Concentration mg/dscm
	Found in Fraction	Blank	Corrected	Total[4]	Found in Fraction	Blank	Corrected	Total[4]		
1	0.45	<0.05	0.45	1.2	1.2	<1	1.2	3.3	4.5	0.21
2	2.1	<0.02	2.1	5.8	<0.8	<0.8	<0.8	<2	5.8	0.27
3	2.1	<0.02	2.1	5.8	1.8	<0.8	1.8	5	11	0.51
4	1.3	<0.02	1.3	3.6	1.8	<0.8	1.8	5	8.6	0.40
5	4.2	<0.02	4.2	12	4.0	<0.8	4	11	23	1.1
6	1.3	<0.02	1.3	3.6	1.4	<0.8	1.4	3.9	7.5	0.35
7	4.6	0.1	4.5	12	41.2	1	40.2	112	124	5.74
Sum	16	0.1	16	44	51	<6	50	140	180	8.58

1. Quantity in entire sample, determined before LC
2. Portion of whole sample used for LC, actual mg
3. Quantity recovered from LC column, actual mg
4. Total mg computed back to total sample

IR REPORT

SAMPLE: 651 Blank XAD F1

Wave Number (cm^{-1})	Intensity	Assignment	Comments
		No Peaks	

IR REPORT

SAMPLE: 651 Blank XAD F2

Wave Number (cm^{-1})	Intensity	Assignment	Comments
		No Peaks	

IR REPORT

SAMPLE: 651 Blank XAD F3

Wave Number (cm^{-1})	Intensity	Assignment	Comments
		No Peaks	

IR REPORT

SAMPLE: 651 Blank XAD F4

Wave Number (cm^{-1})	Intensity	Assignment	Comments
		No Peaks	

IR REPORT

SAMPLE: 651 Blank XAD F5

Wave Number (cm^{-1})	Intensity	Assignment Comments
		No Peaks

IR REPORT

SAMPLE: 651 Blank XAD F6

Wave Number (cm^{-1})	Intensity	Assignment	Comments
		No Peaks	

IR REPORT

SAMPLE: 651 Blank XAD F7

Wave Number (cm^{-1})	Intensity	Assignment	Comments
3600-3300	S	OH	

IR REPORT

SAMPLE: 650 EAI XAD F1

Wave Number (cm^{-1})	Intensity	Assignment Comments
		No Peaks

IR REPORT

SAMPLE: 650 EAI XAD F2

Wave Number (cm^{-1})	Intensity	Assignment	Comments
		No Peaks	

IR REPORT

SAMPLE: 650 EAI XAD F3

Wave Number (cm^{-1})	**Intensity**	**Assignment**	**Comments**
3450	S	OH Aliphatic	

IR REPORT

SAMPLE: 650 EAI XAD F4

Wave Number (cm^{-1})	Intensity	Assignment	Comments
		No Peaks	

IR REPORT

SAMPLE: 650 EAI XAD F5

Wave Number (cm^{-1})	Intensity	Assignment	Comments
		No Peaks	

IR REPORT

SAMPLE: 650 EAI XAD F6

Wave Number (cm^{-1})	Intensity	Assignment	Comments
		No Peaks	

IR REPORT

SAMPLE: 650 EAI XAD F7

Wave Number (cm^{-1})	Intensity	Assignment	Comments
3230	M	OH Carboxylic acid	
2880	M	CH Carboxylic acid	
2810	M	CH Carboxylic acid	
1650	S	C=O Carboxylic acid	
1190	S	CO Carboxylic acid	

SAMPLE: EAII XAD #676

	TCO mg	GRAV mg	TCO + GRAV Total mg	Concentration mg/dscm
Total Sample[1]	20	38	58	2.1
Taken for LC[2]	11	22	33	1.2
Recovered[3]	1.4	12	13	0.48

Fraction	TCO in mg				GRAV in mg				TCO + GRAV Total mg	Concentration mg/dscm
	Found in Fraction	Blank	Corrected	Total[4]	Found in Fraction	Blank	Corrected	Total[4]		
1	0.30	<0.05	0.30	0.53	2.8	<1	2.8	4.9	5.4	0.20
2	<0.02	<0.02	<0.02	<0.04	1	<0.8	1	1.8	1.8	0.07
3	<0.02	<0.02	<0.02	<0.04	<0.8	<0.8	<0.8	<1	<1	<0.04
4	<0.02	<0.02	<0.02	<0.04	1.4	<0.8	1.4	2.5	2.5	0.09
5	<0.02	<0.02	<0.02	<0.04	<0.8	<0.8	<0.8	<1	<1	<0.04
6	0.96* (9.1)	0.02	0.96	1.7	2.4	<0.8	2.4	4.2	5.9	0.22
7	0.12* (4.6)	0.1	<0.1	<0.2	5	1	4.0	7.0	7.0	0.26
Sum	1.4	0.1	1.3	2.2	13	<6	12	21	23	0.84

1. Quantity in entire sample, determined before LC
2. Portion of whole sample used for LC, actual mg
3. Quantity recovered from LC column, actual mg
4. Total mg computed back to total sample

*Corrected for resin contamination -- uncorrected number in parentheses

IR REPORT

SAMPLE: 676 EAII XAD F1

Wave Number (cm^{-1})	Intensity	Assignment Comments
		No Peaks

IR REPORT

SAMPLE: 676 EAII XAD F2

Wave Number (cm^{-1})	Intensity	Assignment	Comments
		No Peaks	

IR REPORT

SAMPLE: 676 EAII XAD F3

Wave Number (cm^{-1})	Intensity	Assignment	Comments
3450	S	OH Aliphatic	

IR REPORT

SAMPLE: 676 EAII XAD F4

Wave Number (cm^{-1})	Intensity	Assignment Comments
		No Peaks

IR REPORT

SAMPLE: 676 EAII XAD F5

Wave Number (cm^{-1})	Intensity	Assignment	Comments
		No Peaks	

IR REPORT

SAMPLE: 676 EAII XAD F6

Wave Number (cm^{-1})	Intensity	Assignment	Comments
		No Peaks	

IR REPORT

SAMPLE: 676 EAII XAD F7

Wave Number (cm^{-1})	Intensity	Assignment	Comments
		No Peaks	

5.9 LOW RESOLUTION MASS SPECTROMETRY (LRMS) OF SELECTED TOTAL SAMPLE EXTRACTS AND LC FRACTIONS

ACUREX Corporation

Energy & Environmental Division

CMEA/ACUREX

December 4, 1981
Acurex ID#: A81-10-011, A81-10-022
Client P.O.#: 307605

ATTENTION: L. Waterland

Samples: 9 extracts from Tosco and Ethan Allen

The above referenced samples were analyzed by direct probe mass spectrometry. Searches have been made for classes of compounds most likely to be found in the various LC fractions, according to procedures described in the "IERL-RTP Procedures Manual: Level 1 Environmental Assessment". The following fragment ions used for search are given below:

Compound Class	Fragment ions (m/e-)
Polycyclic aromatic hydrocarbons	178,202,216,228,252,276
Aliphatic hydrocarbons	57,71
Halogenated aliphatics	79,81,93,95,107,109;49,63
Aromatic hydrocarbons	50,51,77,78,79,91
Ethers	45,59,73
Alcohols	45,59,61,73,75
Phenols	51,77,94
Nitriles	54,68,82
Phthalate esters	61,59,71,87
Amines	44,58
Ketones	51,71
N-heterocyclics	117,167;129,179
Mercaptans, sulfides	47,61,75
Benzothiophenes	57,58,59,69,70,85,97,111,125
Carboxylic acids	60,73,149
Amides	58,72,86,100

To test the analysis procedure, a standard mixture containing ethers, amines, polycyclic aromatic hydrocarbons, nitrosamines, phenols, etc., was analyzed under identical conditions as the samples. Losses of the very volatile compounds such as naphthalene, bis(2-chloroethyl)ether, low molecular weight nitrosamines were observed, however the higher molecular weight compounds in a particular class were recovered.

Prepared by: Greg Nicoll
Greg Nicoll
Program Director

Approved by: Viorica Lopez-Avila
Viorica Lopez-Avila, Ph.D.
Technical Director

GN/VLA:es

LRMS REPORT

SAMPLE: Ethan Allen IXAD 650 F2& F3

Major Categories

Intensity	Category	MW Range
10	Carboxylic acid	
1	PAH	<216

Sub-Categories, Specific Compounds

Intensity	Category	m/e	Composition

Other

LRMS REPORT

SAMPLE: Ethan Allen I XAD 650 F4 & F5

Major Categories

Intensity	Category	MW Range
	None detected	

Sub-Categories, Specific Compounds

Intensity	Category	m/e	Composition

Other

LRMS REPORT

SAMPLE: Ethan Allen I XAD 650 F6 & F7

Major Categories

Intensity	Category	MW Range
	None detected	

Sub-Categories, Specific Compounds

Intensity	Category	m/e	Composition

Other

LRMS REPORT

SAMPLE: Ethan Allen I OMC 652

Major Categories

Intensity	Category	MW Range
100	Ethers	
100	Nitriles	
100	Amines	
100	Heterocyclic sulfur compounds	
100	Carboxylic acids	
10	Halogenated aliphatics	

Sub-Categories, Specific Compounds

Intensity	Category	m/e	Composition

Other

LRMS REPORT

SAMPLE: Ethan Allen I OMC 652 (cont)

Major Categories

Intensity	Category	MW Range
10	Aromatic hydrocarbons	
10	Phenols	
10	Ketones	
10	Heterocyclic nitrogen compounds	
1	PAH	<216

Sub-Categories, Specific Compounds

Intensity	Category	m/e	Composition

Other

LRMS REPORT

SAMPLE: Ethan Allen I Fly Ash 662

Major Categories

Intensity	Category	MW Range
1	PAH	<216

Sub-Categories, Specific Compounds

Intensity	Category	m/e	Composition

Other

LRMS REPORT

SAMPLE: Ethan Allen II XAD 676

Major Categories

Intensity	Category	MW Range
	None detected	

Sub-Categories, Specific Compounds

Intensity	Category	m/e	Composition

Other

LRMS REPORT

SAMPLE: Ethan Allen II Fly Ash 744

Major Categories

Intensity	Category	MW Range
100	Carboxylic acids	
10	PAH	<216

Sub-Categories, Specific Compounds

Intensity	Category	m/e	Composition

Other

Acorn Park
Cambridge, Massachusetts 02140
617 864-5770 Telex 921436

Arthur D. Little, Inc.

July 8, 1982

Dr. Larry Waterland
M2S-2260
Accurex Corporation
485 Clyde Avenue
Mountain View, CA 94042

Dear Larry: 1-7641

We have completed the batch inlet LRMS analysis of your ten Level 1 samples. The data obtained in the analysis of these samples is reported on the enclosed, standard EPA Level 1 LRMS report forms. The intensity levels are reported for this (batch) analysis only, as though it were the complete LRMS analysis. Presumably you will integrate the data from these analyses with your own direct probe LRMS analysis of the same samples.

We have reported the "sample" content of the samples as though the solvent(s) were not present; a component reported as intensity 100 is a major component of the non-solvent portion of the spectrum; one as intensity 10 when it is present and identifiable in the non-solvent portion of the spectrum, and so on. Intensity level 1 components appeared to be present in some of the samples, but were not specifically identifiable. When they occur they are included in the unclassified category.

All samples were analyzed by direct injection of 4 μL of sample into the three liter glass inlet of the mass spectrometer. The mass spectrometer was operated in the electron impact ionization mode, at 70 eV. Low energy ionization was not used due to the low level of sample material as compared to the solvent content of the sample. Instrument blanks were obtained by direct injection of 4 μL of spectra grade methylene chloride. One sample (AC009) was

Brussels Paris Tokyo
Houston Rio de Janeiro Toronto
London San Francisco Washington
Madrid São Paulo Wiesbaden

concentrated 3X and given an additional direct injection analysis. This was done only to clarify some spectral ambiguities. The reported data is from the unconcentrated initial analysis.

If you have any questions about any of this work, please feel free to call me.

Yours Truly,

James L. Stauffer

/laf

enclosures

LRMS REPORT

SAMPLE: Ethan Allen 1 XAD 650 LC2 + LC3 (AC003)

Major Categories

Intensity	Category	MW Range
100	Fused alternate/nonalternate hydrocarbons	<216

Sub-Categories, Specific Compounds

Intensity	Category	m/e	Composition
100	Naphthalene	128	$C_{10}H_8$

Other

LRMS REPORT

SAMPLE: Ethan Allen 1 XAD 650 LC4 + LC5 (AC008)

Major Categories

Intensity	Category	MW Range
100	Aldehydes	106 – 120
10	Aromatic Hydrocarbons	
10	Phenols	94
10	Heterocyclic Oxygen Compounds	118 – 146
10	Unclassified[1]	130

Sub-Categories, Specific Compounds

Intensity	Category	m/e	Composition
100	Benzaldehyde + alkyl	106–120	$C_7H_6O - C_8H_8O$
10	Phenol	94	C_6H_6O
10	Benzofuran + alkyls	118–146	$C_8H_6O - C_{10}H_{10}O$

Other

[1] possibly Heterocyclic Nitrogen Compound (quinoxaline)

LRMS REPORT

SAMPLE: Ethan Allen 1 XAD 650 LC6 & LC7 (AC009)

Major Categories

Intensity	Category	MW Range
100	Phenols	94 - 122
10	Heterocyclic Oxygen Compounds	68 — 146
5	Aldehydes	82 - 124
10	Aromatic Hydrocarbons	

Sub-Categories, Specific Compounds

Intensity	Category	m/e	Composition
100	Phenol and alkyls	94-122	C_6H_6O - $C_8H_{10}O$
5/5	Dimethylfuran / Furaldehyde	96	C_6H_8O / $C_5H_4O_2$
10	Benzofuran + alkyls	118-146	C_8H_6O - $C_{10}H_{10}O$

Other

LRMS REPORT

SAMPLE: Ethan Allen 1 OMC 652 (AC010)

Major Categories

Intensity	Category	MW Range
100	Phenols	94 – 136
10	Heterocyclic Oxygen Compounds	68 – 146
10	Aldehydes	96 – 120
10	Unclassified	

Sub-Categories, Specific Compounds

Intensity	Category	m/e	Composition
100	Phenol + alkyls	94-136	C_6H_6O - $C_9H_{12}O$
10	Benzofuran + alkyls	118-146	C_8H_6O - $C_{10}H_{10}O$
10	Furan + alkyls	68-124	C_4H_4O - $C_8H_{12}O$
10	Furaldehyde + alkyls	96-124	$C_5H_4O_2$ - $C_7H_8O_2$
10	Benzaldehyde + alkyls	106-120	C_7H_6O - C_8H_8O

Other

5.10 RADIOMETRIC ANALYSIS RESULTS

SAFETY SPECIALISTS, Inc.

3284F Edward Avenue, Santa Clara, California 95050 · Telephone (408) 988-1111

ASSAY REPORT

Acurex Corporation
Attn: Mr. Larry Waterland
485 Clyde Avenue
Mountain View, California 94042

Date: August 13, 1981

Date Samples Received: 6/29/81

Customer Order No.: RB59185A, Rel. 15

		Activity*	
SSI No.	Client Description	Gross Alpha pCi/g	Gross Beta pCi/g
81228D	A81-05-030-646 ([illegible])	20.2 ± 12.1	218.8 ± 18.5
E	A81-05-030-662 (Test 1 [illegible])	17.6 ± 4.2	119.0 ± 38.0
F	A81-05-030-674 (Test 2 [illegible] Particulate)	22.2 ± 9.6	164.3 ± 30.5
G	A81-05-030-774 (Test 2 [illegible])	15.6 ± 3.9	93.3 ± 35.0

Analyst: Pamela S. Shreve

TC Noble
Approved: T. C. Noble, Director
Safety and Health Services Division

*The ± values are the two sigma Poisson standard deviation of the counting error.
The ≤ values are equal to or less than three sigma of the counting error.

SAFETY SPECIALISTS, Inc.

3284F Edward Avenue, Santa Clara, California 95050 · Telephone (408) 988-1111

ASSAY REPORT

Acurex Corporation
Attn: Mr. Larry Waterland
485 Clyde Avenue
Mountain View, California 94042

Date: August 13, 1981

Date Samples Received: 6/29/81

Customer Order No.: RB59185A, Rel. 15

SSI No.	Client Description	Activity* Gross Gamma pCi/L	Gross Gamma pCi/g
81228			
D	A81-05-030-646		-415 ± 734
E	A81-05-030-662		4 ± 419
F	A81-05-030-674		161 ± 679
G	A81-05-030-774		163 ± 476

Analyst: Pamela S. Shreve

Approved: T. C. Noble, Director
Safety and Health Services Division

*The ± values are the two sigma Poisson standard deviation of the counting error.
The ≤ values are equal to or less than three sigma of the counting error.

5.11 BIOLOGICAL ASSAY RESULTS

GENETICS ASSAY NO.: 5882
LBI SAFETY NO.: 7166

MUTAGENICITY EVALUATION OF A81-05-030-646 (EA-1 10+3+1+FILTER) IN THE EPA LEVEL 1 AMES SALMONELLA/MICROSOME PLATE TEST

FINAL REPORT

SUBMITTED TO:

ACUREX CORPORATION
485 CLYDE AVENUE
MOUNTAIN VIEW, CALIFORNIA 94042

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20895

LBI PROJECT NO.: 22064

REPORT DATE: NOVEMBER 1981

Litton BIONETICS

PREFACE

This assay conforms to the standard EPA Level 1 procedure for the Ames Salmonella/microsome mutagenesis assay as described in "IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests"[1]. The data were evaluated and formatted as recommended in "Level 1 Biological Testing Assessment and Data Formatting"[2].

The Ames Salmonella/microsome mutagenesis assay has been shown to be a sensitive method for detecting mutagenic activity for a variety of chemicals representing various chemical classes[3]. This assay is one of several recommended by EPA to identify, categorize and rank the pollutant potential of influent and effluent streams from industrial and energy-producing processes. This assay has been well validated with a wide range of positive and negative control chemicals and complex environmental samples.

All procedures and documents pertaining to the receipt, storage, preparation, testing and evaluation of the test material shall conform to Litton Bionetics, Inc. standard operating procedures and the Good Laboratory Practices Regulations of 1979. Deviations from standard procedure shall be fully documented and noted in the report.

All test and control results in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Molecular Toxicology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20895. Copies of raw data will be supplied to the sponsor upon request.

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I. ASSAY SUMMARY

A. Sponsor: Acurex Corporation

B. Material (Test Compound): Genetics Assay Number: 5882

1. Identification: A81-05-030-646 (EA-1 10+3+1+Filter)
2. Date Received: August 26, 1981
3. Physical Description: Fine, brown powder and fiberglass filter with embedded particles.

C. Type of Assay: EPA Level 1 Ames Salmonella/Microsome Plate Test

D. Assay Design Number: 401 (EPA Level 1)

E. Study Dates:

1. Initiation: October 1, 1981
2. Completion: October 26, 1981

F. Supervisory Personnel:

A. Study Director: D.R. Jagannath, Ph.D.

G. Evaluation:

The test material, A81-05-030-646 (EA-1 10+3+1+Filter), was tested for activity in the Ames Salmonella mutagenicity assay over a concentration range of 0.05 mg/plate to 5.0 mg/plate. The test was performed in duplicate under nonactivation and activation test conditions with strains TA-1535, TA-1537, TA-98, and TA-100.

The sample was not mutagenic under the test conditions employed and was ranked as having nondetectable (ND) mutagenic activity as defined by the IERL-EPA Level 1 criteria for the Ames bioassay[1].

Submitted by:

Study Director

D.R. Jagannath 11/24/81

D.R. Jagannath, Ph.D. Date
Section Chief,
Submammalian Genetics,
Department of Molecular
Toxicology

Reviewed by:

11/24/8

David J. Brusick, Ph.D. Date
Director,
Department of Molecular
Toxicology

II. OBJECTIVE

The objective of this study was to determine the genetic activity of A81-05-030-646 (EA-1 10+3+1+filter) in the Salmonella/ microsome assay with and without the addition of mammalian metabolic activation preparations. The genetic activity of a sample is measured in these assays by its ability to revert the Salmonella indicator strains from histidine dependence to histidine independence. The degree of genetic activity of a sample is reflected in the number of revertants that are observed on the histidine-free medium.

III. TEST MATERIAL

A. Description

The test material, as received, was comprised of two separate components. The first component, a fine, brown powder, was the 1 µm, 3 µm and 10 µm SASS train particulate catch. The second component was a fiberglass filter with embedded particulate material. This brown particulate material represented particulates less than 1 µm collected in the SASS train sample. Both components were supplied together in a Nalgene® screw-top bottle.

B. Handling and Preparation

The test material was received at LBI on August 26, 1981. The sample was assigned LBI safety number 7166 and LBI assay number 5882. The sample was stored at +4°C in the dark.

The filter portion of the sample required removal of the embedded particulates before testing could begin. The uncut filter was sonicated in cyclohexane as recommended by current IERL-EPA pretest sample preparation procedures[1]. The decanted particulate suspension from three successive sonication treatments were combined and evaporated to dryness. The particulate material was weighed and combined with the 1 µm particulate catch portion of the sample. A total of 215.14 mg of combined test material available for testing was comprised of 37.78 mg (17.6%) of <1 µm particulates removed from the filter and 177.36 mg (82.4%) of 1 µm, 3 µm and 10 µm particulates.

Appproximately 181 mg of test material were used for the trial in the Ames *Salmonella* Assay. The test material was suspended at 100 mg/ml in dimethylsulfoxide (DMSO) and incubated overnight at 37°C on a rotary shaker. This stock suspension was used to make dilutions in DMSO to be used for dosing in the EPA Level 1 Ames *Salmonella* Assay.

IV. MATERIALS

A. Indicator Microorganisms

The *Salmonella typhimurium* strains used in this assay were obtained from Dr. Bruce Ames, University of California at Berkeley.[4-8] The following four strains were used.

Strain Designation	Gene Affected	Additional Mutations			Mutation Type Detected
		Repair	LPS	R Factor	
TA-1535	his G	Δ uvr B	rfa	-	Base-pair substitution
TA-1537	his C	Δ uvr B	rfa	-	Frameshift
TA-98	his D	Δ uvr B	rfa	pKM101	Frameshift
TA-100	his G	Δ uvr B	rfa	pKM101	Base-pair substitution

All the above strains have, in addition to the mutation in the histidine operon, mutation (rfa-) that leads to defective lipopolysaccharide coat, a deletion that covers genes involved in the synthesis of vitamin biotin (bio-) and in the repair of ultraviolet (uv) - induced DNA damage (uvrB-). The rfa- mutation makes the strains more permeable to many large molecules. The uvrB- mutation decreases repair of some types of chemically or physically damaged DNA and thereby enhances the strain's sensitivity to some mutagenic agents. The resistant transfer factor plasmid (R factor) pKM101 in TA-98 and TA-100 is believed to cause an increase in error-prone DNA repair that leads to many more mutations for a given dose of most mutagens.[8] In addition, plasmid pKM101 confers resistance to the antibiotic ampicillin, which is a convenient marker to detect the presence of plasmid in the cells.

All indicator strains are kept at 4°C on minimal medium plates supplemented with a trace of biotin and an excess of histidine. In addition, the plates with plasmid-carrying strains contain ampicillin (25 μg/ml) to ensure stable maintenance of plasmid pKM101. New stock culture plates are made as often as necessary from the frozen master cultures or from single colony reisolates that were checked for their genotypic characteristics (his, rfa uvrB, bio) and for the presence of plasmid. For each experiment, an inoculum from the stock culture plates is grown overnight at 37°C in nutrient broth (Oxoid CM67) and used.

B. Media

The bacterial strains were cultured in Oxoid Media #2 (Nutrient Broth). The selective medium was Vogen Bonner Medium E with 2% glucose.[10] The

overlay agar consisted of 0.6% purified agar with 0.05 mM histidine, 0.05 mM biotin and 0.1M NaCl according to the methods of Ames *et al.*[9]

C. Activation System

1. S9 Homogenate

A 9,000 x *g* supernatant prepared from Sprague-Dawley adult male rat liver induced by Aroclor 1254 (Ames *et al.*[9]) was purchased commercially and used in these assays.

2. S9 Mix

S9 mix used in these assays consisted of the following components:

Components	Concentration per Milliliter S9 Mix
NADP (sodium salt)	4 µmoles
D-glucose-6-phosphate	5 µmoles
$MgCl_2$	8 µmoles
KCl	33 µmoles
Sodium phosphate buffer pH 7.4	100 µmoles
Organ homogenate from rat liver (S9 fraction)	100 µliters

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V. EXPERIMENTAL DESIGN

A. Dosage Selection

Test strategy and dose selection depend upon sample type and sample availability. The Level 1 manual[1] recommends solids to be initially tested at the maximum applicable dose (MAD) of 5 mg per plate and at lower concentrations of 2.5, 1, 0.5, 0.1 and 0.05 mg per plate. Liquids are tested initially at the MAD of 200 µl per plate, and at lower concentrations of 100, 50 and 10 µl per plate. Samples are retested over a narrower range of concentrations with strains showing positive results initially. Alternate dose are employed if sample size is limiting or at the direction of the sponsor.

Doses selected to test this sample covered the recommended dose range for solids. The highest dose was at the MAD level of 5 mg per plate and included five lower dose levels of 2.5, 1, 0.5, 0.1 and 0.05 mg per plate.

B. Mutagenicity Testing

The procedure used was based on the paper published by Ames et. al.[9] and was performed as follows:

1. Nonactivation Assay

To a sterile 13 x 100 mm test tube placed in a 43°C water bath the following was added in order:

- 2.00 ml of 0.6% agar containing 0.05 mM histidine and 0.05 mM biotin.
- 0.05 ml of a suspension of the test chemical to give the appropriate dose.
- 0.1 ml to 0.2 ml of indicator organism(s).
- 0.50 ml of 0.2M phosphate buffer, pH 7.4.

This mixture was swirled gently and then poured onto minimal agar plates (see IV B, Media). After the top agar had set, the plates were incubated at 37°C for approximately 2 days. The number of his+ revertant colonies growing on the plates were counted with an automatic colony counter and recorded.

2. Activation Assay

The activation assay was run concurrently with the nonactivation assay. The only difference was the addition of 0.5 ml of S9 mix (see IV C, Activation System) to the tubes in place of 0.5 ml of phosphate buffer which was added in nonactivation assays. All other details were similar to the procedure for nonactivation assays.

A detailed flow diagram for the plate incorporation assay is provided in Figure 1.

C. Control Compounds

A negative control consisting of the solvent used for the test material was also assayed concurrently with the test material. For negative controls, step 'b' of Nonactivation Assays was replaced by 0.05 ml of the solvent. The negative controls were employed for each indicator strain and were performed in the absence and presence of S9 mix. The solvent used to prepare the stock solution of the test material is given in the Results section of this report. All dilutions of the test material were made using this solvent. The amount of solvent used was equal to the maximum volume used to give the appropriate test dose.

Specific positive control compounds known to revert each strain were also used and assayed concurrently with the test material. The concentrations and specificities of these compounds to specific strains are given in the following table:

Assay	Chemical	Solvent	Concentration per plate (μg)	Salmonella Strains
Nonactivation	Sodium azide	Water	10.0	TA-1535, TA-100
	2-Nitrofluorene (NF)	Dimethylsulfoxide	10.0	TA-98
	9-aminoacridine (9AA)	Ethanol	50.0	TA-1537
Activation	2-anthramine (ANTH)	Dimethylsulfoxide	2.5	For all strains

D. Recording and Presenting Data

The number of colonies on each plate were counted and recorded on printed forms. These raw data were analyzed in a computer program and reported on a printout. The results are presented as revertants per plate for each indicator strain employed in the assay. The positive and solvent controls are provided as reference points.

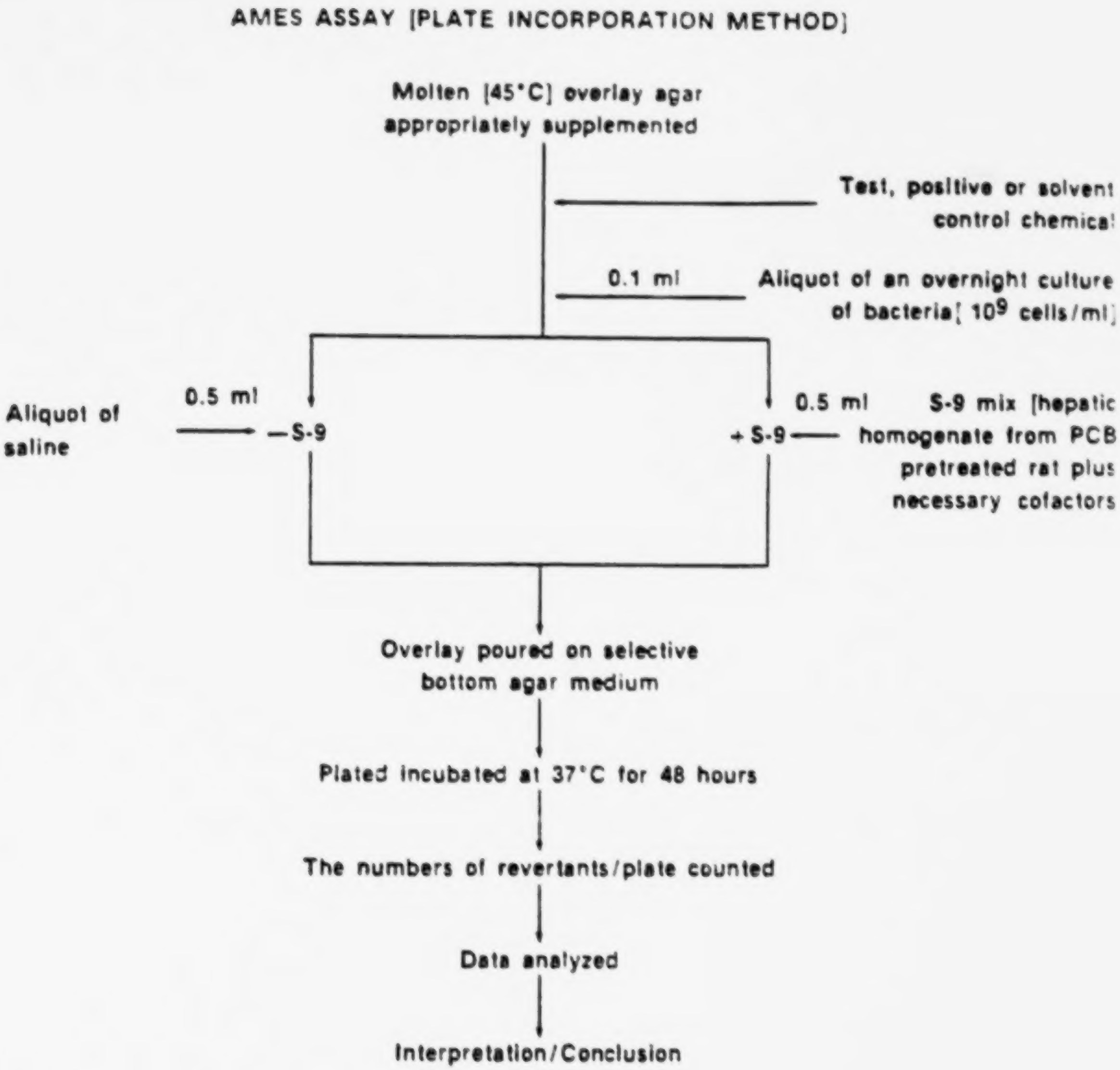
AMES ASSAY [PLATE INCORPORATION METHOD]
Molten [45°C] overlay agar appropriately supplemented
Test, positive or solvent control chemical
0.1 ml
Aliquot of an overnight culture of bacteria [10^9 cells/ml]
Aliquot of saline
0.5 ml
−S-9
0.5 ml
+S-9
S-9 mix [hepatic homogenate from PCB pretreated rat plus necessary cofactors
Overlay poured on selective bottom agar medium
Plated incubated at 37°C for 48 hours
The numbers of revertants/plate counted
Data analyzed
Interpretation/Conclusion

Figure 1 AMES SALMONELLA/MICROSOME MUTAGENESIS ASSAY

VI. RESULTS

A. Interpretations

The test material, A81-05-030-646 (EA-1 10+3+1+filter), was dissolved in DMSO at a stock concentration of 100 mg/ml and leached overnight on a shaker at 37°C. Additional dilutions were prepared in DMSO for testing. The maximum test level was 5.0 mg/plate except for the activation portion of the assay with strain TA-1535 which used a maximum dose of 1 mg/plate because of limited test material. There was no evidence of toxicity at this level.

Reverse mutation was measured in strains TA-1535, TA-1537, TA-98 and TA-100. The test was conducted in duplicate both with and without rat liver S9 mix for metabolic activation.

There was no mutagenic activity associated with the test material treatment and the sample was considered nonmutagenic and non toxic. The sample was ranked as having nondetectable (ND) mutagenic activity using the IERL-EPA Level 1 evaluation criteria for the Ames Assay[1].

Solvent control and positive control values were within acceptable ranges. These results achieved assay acceptance criteria and provided confidence in the assumptions that the recorded data represented typical responses to the test material.

B. Tables

This report is based on the data provided in Table 1.

RESULTS

TABLE 1

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: A-81-05-030-646 (EA-1 10+3+1+FILTER)
B. SOLVENT: DMSO
C. TEST INITIATION DATES: 10/22/81
D. TEST COMPLETION DATE: 10/26/81
E. S-9 LOT#: REF050
NOTE: CONCENTRATIONS ARE GIVEN IN MILLIGRAMS PER PLATE

TEST		SPECIES	TISSUE	REVERTANTS PER PLATE											
				TA-1535			TA-1537			TA-98			TA-100		
				1	2	3	1	2	3	1	2	3	1	2	3
NONACTIVATION															
SOLVENT CONTROL		---	---	8	10		7	8		26	20		119	104	
POSITIVE CONTROL**		---	---	1021	1034		117	190		552	845		1543	1515	
TEST COMPOUND															
0.050	MG	---	---	15	14		7	7		21	35		124	132	
0.100	MG	---	---	18	15		4	6		13	25		117	141	
0.500	MG	---	---	19	15		10	5		28	30		112	132	
1.000	MG	---	---	19	18		8	5		30	32		121	128	
2.500	MG	---	---	20	23		9	4		32	25		116	121	
5.000	MG	---	---	29	20		7	7		22	25		102	126	
ACTIVATION															
SOLVENT CONTROL		RAT	LIVER	11	10		13	16		37	24		94	101	
POSITIVE CONTROL***		RAT	LIVER	140	145		178	187		810	950		1586	1833	
TEST COMPOUND															
0.050	MG	RAT	LIVER	6	10		10	13		35	35		95	129	
0.100	MG	RAT	LIVER	12	9		6	5		43	49		100	116	
0.500	MG	RAT	LIVER	17	7		11	12		37	38		126	121	
1.000	MG	RAT	LIVER	C	14		14	4		27	34		100	90	
2.500	MG	RAT	LIVER	-	-		17	7		28	28		107	122	
5.000	MG	RAT	LIVER	-	-		10	10		29	37		93	103	

**

TA-1535	SODIUM AZIDE	10 UG/PLATE
TA-1537	9-AMINOACRIDINE	50 UG/PLATE
TA-98	2-NITROFLUORENE	10 UG/PLATE
TA-100	SODIUM AZIDE	10 UG/PLATE
SOLVENT	50 UL/PLATE	

***

TA-1535	2-ANTHRAMINE	2.5 UG/PLATE
TA-1537	2-ANTHRAMINE	2.5 UG/PLATE
TA-98	2-ANTHRAMINE	2.5 UG/PLATE
TA-100	2-ANTHRAMINE	2.5 UG/PLATE

- INDICATES TEST WAS NOT DONE

C INDICATES CONTAMINATION

VII. ASSAY ACCEPTANCE AND EVALUATION CRITERIA

Statistical methods are not currently used, and evaluation is based on the criteria included in this protocol.

Plate test data consists of direct revertant colony counts obtained from a set of selective agar plates seeded with populations of mutant cells suspended in a semisolid overlay. Because the test material and the cells are incubated in the overlay for approximately 2 days and a few cell divisions occur during the incubation period, the test is semiquantitative in nature. Although these features of the assay reduce the quantitation of results, they provide certain advantages not contained in a quantitative suspension test:

- The small number of cell divisions permits potential mutagens to act on replication DNA, which is often more sensitive than nonreplicating DNA.
- The combined incubation of the test article and the cells in the overlay permits constant exposure of the indicator cells for approximately 2 days.

A. Surviving Populations

Plate test procedures do not permit exact quantitation of the number of cells surviving chemical treatment. At low concentrations of the test material, the surviving population on the treatment plates is essentially the same as that on the negative control plate. At high concentrations, the surviving population is usually reduced by some fraction. Our protocol will normally employ several doses ranging over two or three log concentrations, the highest of these doses being selected to show slight toxicity as determined by subjective criteria.

B. Dose-Response Phenomena

The demonstration of dose-related increased in mutant counts is an important criterion in establishing metagenicity. A factor that might modify dose-response results for a mutagen would be the selection of doses that are too low (usually mutagenicity and toxicity are related). If the highest dose is far lower than a toxic concentration, no increases may be observed over the dose range selected. Conversely, if the lowest dose employed is highly cytotoxic, the test material may kill any mutants that are induced, and the test material will not appear to be mutagenic.

C. Control Tests

Positive and negative control assays were conducted with each experiment and consisted of direct-acting mutagens for nonactivation assays and mutagens that require metabolic biotransformation in activation assays.

Negative controls consisted of the test material solvent in the overlay agar together with the other essential components. The negative control plate for each strain gave a reference point to which the test data was compared. The positive control assay was conducted to demonstrate that the test systems were functional with known mutagens.

The following normal range of revertants for solvent controls are generally considered acceptable.

TA-1535:	8-30
TA-1537:	4-30
TA-98:	20-75
TA-100:	80-250

D. Evaluation Criteria for Ames Assay

Because the procedures to be used to evaluate the mutagenicity of the test material are semiquantitative, the criteria to be used to determine positive effects are inherently subjective and are based primarily on a historical data base. Most data sets will be evaluated using the following criteria.

1. Strains TA-1535 and TA-1537

If the solvent control value is within the normal range, a test material that produces a positive dose response over three concentrations with the highest increase equal to three times the solvent control value will be considered to be mutagenic.

2. Strains TA-98 and TA-100

If the solvent control value is within the normal range, a test material that produces a positive dose response over three concentrations with the highest increase equal to twice the solvent control value for TA-98 and TA-100 will be considered to be mutagenic.

3. Pattern

Because TA-1535 and TA-100 are both derived from the same parental strain (G-46), to some extent there is a built-in redundancy in the microbial assay. In general, the two strains of a set respond to the same mutagen and such a pattern is sought. Generally, if a strain responds to a mutagen in nonactivation tests, it will do so in activation tests.

4. Reproducibility

If a test material produces a response in a single test that cannot be reproduced in additional runs, the initial positive test data lose significance.

The preceding criteria are not absolute, and other extenuating factors may enter into a final evaluation decision. However, these criteria will be applied to the majority of situations and are presented to aid those individuals not familar with this procedure. As the data base is increased, the criteria for evaluation can be more firmly established.

E. Relation Between Mutagenicity and Carcinogenicity

It must be emphasized that the Ames *Salmonella*/Microsome Plate Assay is not a definitive test for chemical carcinogens. It is recognized, however, that correlative and functional relations have been demonstrated between these two endpoints. The results of comparative tests on 300 chemicals by McCann *et al.*[4] show an extremely good correlation between results of microbial mutagenesis tests and *in vivo* rodent carcinogenesis assays.

All evaluations and interpretation of the data to be presented in the final report will be based only on the demonstration, or lack, of mutagenic activity.

F. Criteria for Ranking Samples in the Ames Assay

The goal of EPA Level 1 Ames testing is to rank source streams by relative degree of genetic toxicity (mutagenicity). Samples are first identified as mutagenic or nonmutagenic by the criteria in Section D above and then ranked using the mutagenicity categories presented in the table below. The lowest concentration giving a positive response in any strain, with or without metabolic activation, is identified as the minimum effective concentration (MEC) for that sample. The mutagenicity of the sample is evaluated as high (H), moderate (M), low (L), or nondetectable (ND) according to the evaluation criteria developed in the Level 1 manual[1] and summarized below. Samples with no detectable activity at the maximum applicable dose (MAD) are ranked nondetectable (ND).

Ames Assay Mutagenicity Ranking Criteria[1]

Mutagenic Activity	Solids (MEC in μg/plate)	Liquids[a] (MEC in μl/plate)
High (H)	<50	<2
Moderate (M)	50-500	2-20
Low (L)	500-5000	20-200
Not Detectable (ND)	>5000	>200

[a] Concentration of organic extracts is based upon organic content (μg organics per plate) and not volume (μl extract per plate) of sample tested.

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GENETICS ASSAY NO.: 5879
LBI SAFETY NO.: 7163

MUTAGENICITY EVALUATION OF
A81-05-030-650
(EA-1 XAD EXTRACT)
IN THE
EPA LEVEL 1
AMES SALMONELLA/MICROSOME
PLATE TEST

FINAL REPORT

SUBMITTED TO:

ACUREX CORPORATION
485 CLYDE AVENUE
MOUNTAIN VIEW, CALIFORNIA 94042

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20895

LBI PROJECT NO.: 22064

REPORT DATE: NOVEMBER 1981

Litton BIONETICS

PREFACE

This assay conforms to the standard EPA Level 1 procedure for the Ames *Salmonella*/microsome mutagenesis assay as described in "IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests"[1]. The data were evaluated and formatted as recommended in "Level 1 Biological Testing Assessment and Data Formatting"[2].

The Ames *Salmonella*/microsome mutagenesis assay has been shown to be a sensitive method for detecting mutagenic activity for a variety of chemicals representing various chemical classes[3]. This assay is one of several recommended by EPA to identify, categorize and rank the pollutant potential of influent and effluent streams from industrial and energy-producing processes. This assay has been well validated with a wide range of positive and negative control chemicals and complex environmental samples.

All procedures and documents pertaining to the receipt, storage, preparation, testing and evaluation of the test material shall conform to Litton Bionetics, Inc. standard operating procedures and the Good Laboratory Practices Regulations of 1979. Deviations from standard procedure shall be fully documented and noted in the report.

All test and control results in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Molecular Toxicology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20895. Copies of raw data will be supplied to the sponsor upon request.

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I. ASSAY SUMMARY

A. Sponsor: Acurex Corporation

B. Material (Test Compound): Genetics Assay Number: 5879

1. Identification: A81-05-030-650 (EA-1 XAD Extract)
2. Date Received: August 26, 1981
3. Physical Description: Clear, amber/brown liquid.

C. Type of Assay: EPA Level 1 Ames Salmonella/Microsome Plate Test

D. Assay Design Number: 401 (EPA Level 1)

E. Study Dates:

1. Initiation: September 23, 1981
2. Completion: October 16, 1981

F. Supervisory Personnel:

A. Study Director: D.R. Jagannath, Ph.D.

G. Evaluation:

The test material, A81-05-030-650 (EA-1 XAD extract), contained 18.3 mg organics per ml after solvent exchange into dimethylsulfoxide (DMSO). The solvent exchanged sample was evaluated for its genetic activity in the EPA Level 1 Ames assay, directly, and in the presence of S9 metabolic activation mix. The test sample was mutagenic to TA-1537, TA-98 and TA-100 in the activation and nonactivation assays. The tests indicate that the test material contains both base-pair and frameshift type mutagens. The dose-related mutagenic response was observed at a minimum concentration of 2.5 µl (or 45.75 µg organics) per plate with TA-1537 and TA-98 in the activation assays. The MEC of 45.75 µg/plate, while in the high mutagenicity category, closely approached the high/moderate boundary. The test material, therefore, was ranked as having high/moderate (H/M) borderline mutagenicity using the IERL-EPA Level 1 evaluation criteria for the Ames Assay[1].

Submitted by:

Study Director

D.R. Jagannath 11/24/81

D.R. Jagannath, Ph.D. Date
Section Chief,
Submammalian Genetics,
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Reviewed by:

11/24/81

David J. Brusick, Ph.D. Date
Director,
Department of Molecular
Toxicology

II. OBJECTIVE

The objective of this study was to determine the genetic activity of A81-05-030-650 (EA-1 XAD Extract) in the *Salmonella*/ microsome assay with and without the addition of mammalian metabolic activation preparations. The genetic activity of a sample is measured in these assays by its ability to revert the *Salmonella* indicator strains from histidine dependence to histidine independence. The degree of genetic activity of a sample is reflected in the number of revertants that are observed on the histidine-free medium.

III. TEST MATERIAL

A. Description

The test material was received as a clear, amber-brown solution in methylene chloride. The sample contained 75.0 milligrams of organic material in 0.7 ml of methylene chloride. No information on the sampling parameters (such as the equivalent volume of stack gas represented by the sample) was provided.

B. Handling and Preparation

The test material was received at LBI on August 26, 1981. The sample was assigned LBI safety number 7163 and LBI assay number 5879. The sample was stored at +4°C in the dark.

Pretest sample preparation consisted of solvent exchanging the sample into dimethylsulfoxide (DMSO). The sample was transferred with methylene chloride rinses into a graduated conical tube. The methylene cholride was gradually evaporated (50°C under a stream of nitrogen) and DMSO was sequentially added. The sample was brought to volume in 4.1 ml of DMSO, giving a sample concentration of 18.3 mg organics per ml DMSO. The sample was transferred to a glass vial and sealed with a teflon-coated rubber septum.

Approximately 3.0 ml of test material was used for testing in two trials. Varying aliquots of the test material were added directly to the test mixtures to give the desired concentration. The amount of sample used in Trial 1 was 2.9 ml and 75 μl was used in Trial 2.

IV. MATERIALS

A. Indicator Microorganisms

The Salmonella typhimurium strains used in this assay were obtained from Dr. Bruce Ames, University of California at Berkeley.[4-8] The following four strains were used.

Strain Designation	Gene Affected	Additional Mutations			Mutation Type Detected
		Repair	LPS	R Factor	
TA-1535	his G	Δ uvr B	rfa	-	Base-pair substitution
TA-1537	his C	Δ uvr B	rfa	-	Frameshift
TA-98	his D	Δ uvr B	rfa	pKM101	Frameshift
TA-100	his G	Δ uvr B	rfa	pKM101	Base-pair substitution

All the above strains have, in addition to the mutation in the histidine operon, mutation (rfa-) that leads to defective lipopolysaccharide coat, a deletion that covers genes involved in the synthesis of vitamin biotin (bio-) and in the repair of ultraviolet (uv) - induced DNA damage (uvrB-). The rfa- mutation makes the strains more permeable to many large molecules. The uvrB- mutation decreases repair of some types of chemically or physically damaged DNA and thereby enhances the strain's sensitivity to some mutagenic agents. The resistant transfer factor plasmid (R factor) pKM101 in TA-98 and TA-100 is believed to cause an increase in error-prone DNA repair that leads to many more mutations for a given dose of most mutagens.[8] In addition, plasmid pKM101 confers resistance to the antibiotic ampicillin, which is a convenient marker to detect the presence of plasmid in the cells.

All indicator strains are kept at 4°C on minimal medium plates supplemented with a trace of biotin and an excess of histidine. In addition, the plates with plasmid-carrying strains contain ampicillin (25 µg/ml) to ensure stable maintenance of plasmid pKM101. New stock culture plates are made as often as necessary from the frozen master cultures or from single colony reisolates that were checked for their genotypic characteristics (his, rfa uvrB, bio) and for the presence of plasmid. For each experiment, an inoculum from the stock culture plates is grown overnight at 37°C in nutrient broth (Oxoid CM67) and used.

B. Media

The bacterial strains were cultured in Oxoid Media #2 (Nutrient Broth). The selective medium was Vogen Bonner Medium E with 2% glucose.[10] The

overlay agar consisted of 0.6% purified agar with 0.05 mM histidine, 0.05 mM biotin and 0.1M NaCl according to the methods of Ames et al.[9]

C. Activation System

1. S9 Homogenate

A 9,000 x g supernatant prepared from Sprague-Dawley adult male rat liver induced by Aroclor 1254 (Ames et al.[9]) was purchased commercially and used in these assays.

2. S9 Mix

S9 mix used in these assays consisted of the following components:

Components	Concentration per Milliliter S9 Mix
NADP (sodium salt)	4 µmoles
D-glucose-6-phosphate	5 µmoles
$MgCl_2$	8 µmoles
KCl	33 µmoles
Sodium phosphate buffer pH 7.4	100 µmoles
Organ homogenate from rat liver (S9 fraction)	100 µliters

V. EXPERIMENTAL DESIGN

A. Dosage Selection

Test strategy and dose selection depend upon sample type and sample availability. The Level 1 manual[1] recommends solids to be initially tested at the maximum applicable dose (MAD) of 5 mg per plate and at lower concentrations of 2.5, 1, 0.5, 0.1 and 0.05 mg per plate. Liquids are tested initially at the MAD of 200 µl per plate, and at lower concentrations of 100, 50 and 10 µl per plate. Samples are retested over a narrower range of concentrations with strains showing positive results initially. Alternate dose are employed if sample size is limiting or at the direction of the sponsor.

Doses selected for the initial test of sample covered the recommended dose range for liquid samples. The highest dose was at the MAD level of 200 µl/ml per plate and included three lower dose levels of 100, 50 and 10 µl per plate. These dose levels corresponded to 3660, 1830, 915, and 183 µg organics per plate. The second trial, using a lower range of doses, was conducted using dose levels of 5, 2.5 and 1.0 µl per plate. These doses corresponded to 91.5, 45.75 and 18.3 µg organics per plate.

B. Mutagenicity Testing

The procedure used was based on the paper published by Ames *et*. *al*.[9] and was performed as follows:

1. Nonactivation Assay

To a sterile 13 x 100 mm test tube placed in a 43°C water bath the following was added in order:

- 2.00 ml of 0.6% agar containing 0.05 mM histidine and 0.05 mM biotin.
- 0.01 ml to 0.2 ml of a solution of the test chemical to give the appropriate dose.
- 0.1 ml to 0.2 ml of indicator organism(s).
- 0.50 ml of 0.2M phosphate buffer, pH 7.4.

This mixture was swirled gently and then poured onto minimal agar plates (see IV B, Media). After the top agar had set, the plates were incubated at 37°C for approximately 2 days. The number of *his*+ revertant colonies growing on the plates were counted with an automatic colony counter and recorded.

2. Activation Assay

The activation assay was run concurrently with the nonactivation assay. The only difference was the addition of 0.5 ml of S9 mix (see IV C, Activation System) to the tubes in place of 0.5 ml of phosphate buffer which was added in nonactivation assays. All other details were similar to the procedure for nonactivation assays.

A detailed flow diagram for the plate incorporation assay is provided in Figure 1.

C. Control Compounds

A negative control consisting of the solvent used for the test material was also assayed concurrently with the test material. For negative controls, step 'b' of Nonactivation Assays was replaced by 0.05 ml of the solvent. The negative controls were employed for each indicator strain and were performed in the absence and presence of S9 mix. The solvent used to prepare the stock solution of the test material is given in the Results section of this report. All dilutions of the test material were made using this solvent. The amount of solvent used was equal to the maximum volume used to give the appropriate test dose.

Specific positive control compounds known to revert each strain were also used and assayed concurrently with the test material. The concentrations and specificities of these compounds to specific strains are given in the following table:

Assay	Chemical	Solvent	Concentration per plate (μg)	Salmonella Strains
Nonactivation	Sodium azide	Water	10.0	TA-1535, TA-100
	2-Nitrofluorene (NF)	Dimethyl-sulfoxide	10.0	TA-98
	9-aminoacridine (9AA)	Ethanol	50.0	TA-1537
Activation	2-anthramine (ANTH)	Dimethyl-sulfoxide	2.5	For all strains

D. Recording and Presenting Data

The number of colonies on each plate were counted and recorded on printed forms. These raw data were analyzed in a computer program and reported on a printout. The results are presented as revertants per plate for each indicator strain employed in the assay. The positive and solvent controls are provided as reference points.

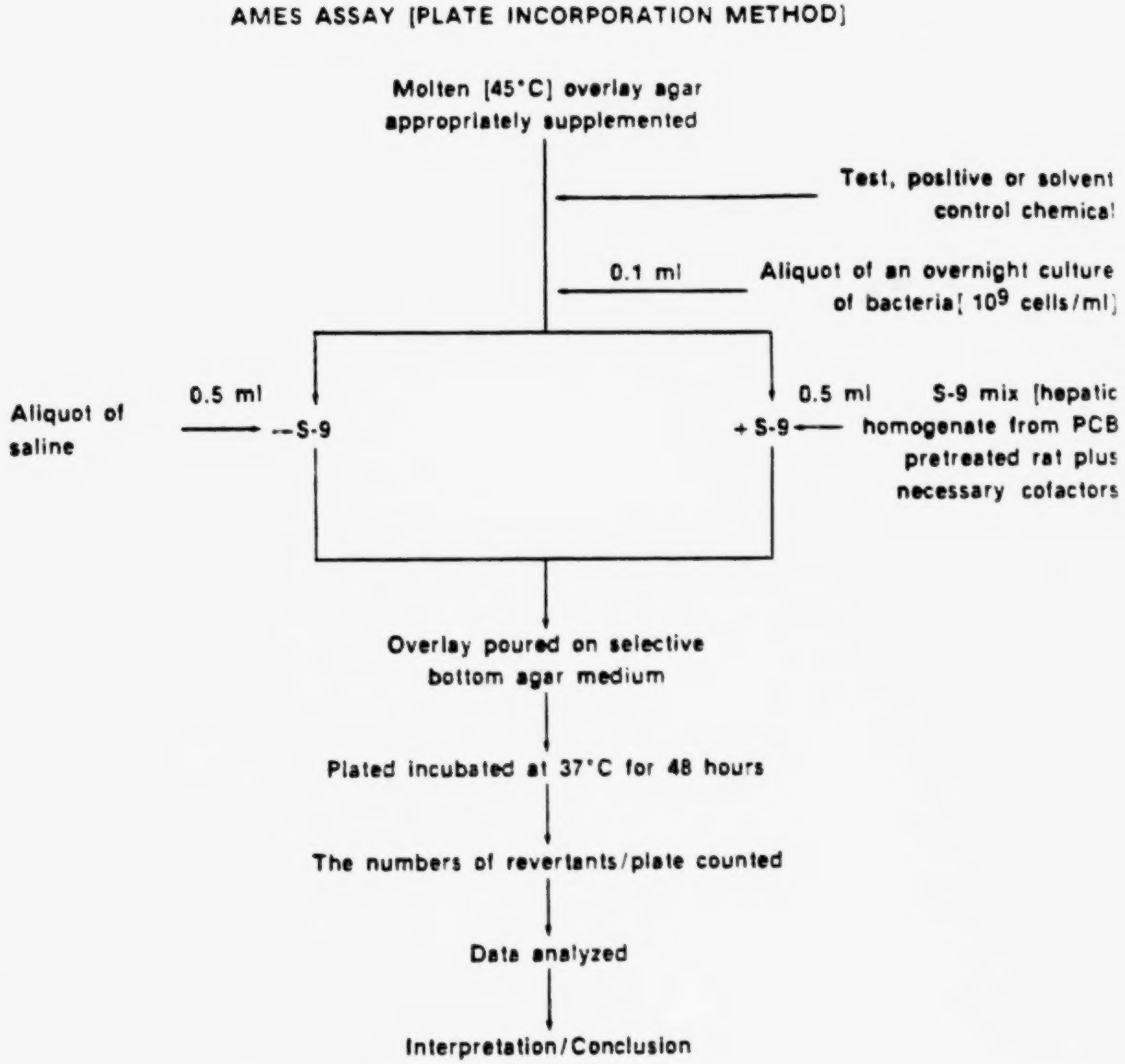
AMES ASSAY [PLATE INCORPORATION METHOD]
Molten [45°C] overlay agar appropriately supplemented
Test, positive or solvent control chemical
0.1 ml
Aliquot of an overnight culture of bacteria [10^9 cells/ml]
Aliquot of saline
0.5 ml
−S-9
0.5 ml
+S-9
S-9 mix [hepatic homogenate from PCB pretreated rat plus necessary cofactors
Overlay poured on selective bottom agar medium
Plated incubated at 37°C for 48 hours
The numbers of revertants/plate counted
Data analyzed
Interpretation/Conclusion

Figure 1 AMES SALMONELLA/MICROSOME MUTAGENESIS ASSAY

VI. RESULTS

A. Interpretations

The test material, A81-05-030-650 (EA-1 XAD extract), in methylene chloride was solvent exchanged to DMSO and this solvent exchanged material was tested for its genetic activity in the EPA Level 1 Ames assays. The organic content, after solvent exchange, was 18.3 mg per ml. Initially, the test was performed only with TA-98 and TA-100 at four dose levels from 10.0 µl per plate to 200.0 µl per plate doses due to the limited quantity of the test sample.

The initial results with TA-98 and TA-100 exhibited positive response at the lowest dose of 10.0 µl per plate with both strains. The test sample was toxic to both strains at doses of 50.0 µl and above in the nonactivation assays. As such, repeat tests were conducted using all the four Salmonella strains at 1, 2.5 and 5.0 µl/plate in the activation and nonactivation assays.

The repeat tests conducted on the test sample were positive with TA-1537 and TA-98 in the activation and nonactivation assays and with TA-100 in the activation assays. The minimum effective concentration that exhibited the mutagenic response was at 2.5 µl per plate (45.75 µg organics/plate) in the activation assays with TA-1537 and TA-98. This response, while in the high mutagenicity category, closely approached the high/moderate borderline. The test material, therefore, was ranked as having high/moderate (H/M) mutagenicity using the IERL-EPA Level 1 evaluation criteria for the Ames Assay[1]. These tests indicate that the XAD extract of the test material, A81-05-030-650 (EA-1 XAD extract), contains both base-pair and frameshift type mutagens.

Solvent control and positive control values were within acceptable ranges. These results achieved assay acceptance criteria and provided confidence in the assumptions that the recorded data represented typical responses to the test material.

B. Tables

This report is based on the data provided in Tables 1 and 2.

```
  RESULTS                                          TABLE 1
--------------------------------

A.    NAME OR CODE DESIGNATION OF THE TEST COMPOUND:  A81-05-030-650 (EA-1 XAD EXTRACT)
B.    SOLVENT:  NONE
C.    TEST INITIATION DATES:  10/01/81
D.    TEST COMPLETION DATE: 10/05/81
E.    S-9 LOT#: REF050
NOTE:   CONCENTRATIONS ARE GIVEN IN MICROLITERS   PER  PLATE

                                    R E V E R T A N T S   P E R   P L A T E
                                  -------------------------------------------------------------------------
TEST               SPECIES TISSUE  TA-98           TA-100
----               ------- ------ --------------- --------------
                                     1    2    3    1    2    3
NONACTIVATION
-------------


SOLVENT CONTROL      ---     ---    30   30        134  128
POSITIVE CONTROL**   ---     ---   760  814       1192 1363

TEST COMPOUND
     10.00      UL   ---     ---    93  119        303  249
     50.00      UL   ---     ---     0    0          0    0
    100.00      UL   ---     ---     0    0          0    0
    200.00      UL   ---     ---     0    0          0    0

ACTIVATION
----------


SOLVENT CONTROL      RAT    LIVER   38   45        127  132
POSITIVE CONTROL***  RAT    LIVER 2036            2074 2145

TEST COMPOUND
     10.00      UL   RAT    LIVER  466  301        397  345
     50.00      UL   RAT    LIVER  315  218        351  348
    100.00      UL   RAT    LIVER  185  110          0    0
    200.00      UL   RAT    LIVER    0    0          0    0
-------------------------------------------------------------------------------------------------------------

**                                                            ***
   TA-98    2-NITROFLUORENE                    10 UG/PLATE        TA-98     2-ANTHRAMINE  2.5 UG/PLATE
   TA-100   SODIUM AZIDE                       10 UG/PLATE        TA-100    2-ANTHRAMINE  2.5 UG/PLATE
   SOLVENT  50 UL/PLATE
 - INDICATES TEST WAS NOT DONE
```

RESULTS

TABLE 2

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUNC: A81-05-03C-650 (EA-1XAD EXTRACT)
B. SCLVENT: DMSC
C. TEST INITIATION DATES: 10/13/81
D. TEST COMPLETION DATE: 10/16/81
E. S-9 LOT#: REF050
NCTE: CONCENTRATIONS ARE GIVEN IN MICROLITERS PER PLATE

TEST		SPECIES	TISSUE	REVERTANTS FER FLATE											
				TA-1535			TA-1537			TA-98			TA-100		
				1	2	3	1	2	3	1	2	3	1	2	3
NONACTIVATION															
SOLVENT CONTROL		---	---	12			9			27			123		
SOLVENT CONTROL		---	---	16			9			22			109		
PCSITIVE CONTROL**		---	---	758			256			958			1404		
POSITIVE CONTROL**		---	---	1007			220			933			1370		
TEST COMPOUND															
1.00	UL	---	---	14			7			38			112		
2.50	UL	---	---	19			12			35			139		
5.00	UL	---	---	8			28			50			191		
ACTIVATION															
SOLVENT CONTROL		RAT	LIVER	8			6			38			97		
SOLVENT CONTROL		RAT	LIVER	11			7			37			105		
PCSITIVE CONTROL***		RAT	LIVER	391			431			1680			2113		
POSITIVE CONTROL***		RAT	LIVER	351			416			1758			1997		
TEST COMPCUND															
1.00	UL	RAT	LIVER	10			15			62			134		
2.50	UL	RAT	LIVER	11			27			111			173		
5.00	UL	RAT	LIVER	13			46			170			249		

**

TA-1535	SCDIUM AZIDE	10 UG/PLATE
TA-1537	9-AMINOACRIDINE	50 UG/PLATE
TA-98	2-NITROFLUORENE	10 UG/PLATE
TA-100	SODIUM AZIDE	10 UG/PLATE
SOLVENT	50 UL/PLATE	

***

TA-1535	2-ANTHRAMINE	2.5 UG/PLATE
TA-1537	2-ANTHRAMINE	2.5 UG/PLATE
TA-98	2-ANTHRAMINE	2.5 UG/PLATE
TA-100	2-ANTHRAMINE	2.5 UG/PLATE

- INDICATES TEST WAS NOT DONE

VII. ASSAY ACCEPTANCE AND EVALUATION CRITERIA

Statistical methods are not currently used, and evaluation is based on the criteria included in this protocol.

Plate test data consists of direct revertant colony counts obtained from a set of selective agar plates seeded with populations of mutant cells suspended in a semisolid overlay. Because the test material and the cells are incubated in the overlay for approximately 2 days and a few cell divisions occur during the incubation period, the test is semiquantitative in nature. Although these features of the assay reduce the quantitation of results, they provide certain advantages not contained in a quantitative suspension test:

- The small number of cell divisions permits potential mutagens to act on replication DNA, which is often more sensitive than nonreplicating DNA.
- The combined incubation of the test article and the cells in the overlay permits constant exposure of the indicator cells for approximately 2 days.

A. Surviving Populations

Plate test procedures do not permit exact quantitation of the number of cells surviving chemical treatment. At low concentrations of the test material, the surviving population on the treatment plates is essentially the same as that on the negative control plate. At high concentrations, the surviving population is usually reduced by some fraction. Our protocol will normally employ several doses ranging over two or three log concentrations, the highest of these doses being selected to show slight toxicity as determined by subjective criteria.

B. Dose-Response Phenomena

The demonstration of dose-related increased in mutant counts is an important criterion in establishing metagenicity. A factor that might modify dose-response results for a mutagen would be the selection of doses that are too low (usually mutagenicity and toxicity are related). If the highest dose is far lower than a toxic concentration, no increases may be observed over the dose range selected. Conversely, if the lowest dose employed is highly cytotoxic, the test material may kill any mutants that are induced, and the test material will not appear to be mutagenic.

C. Control Tests

Positive and negative control assays were conducted with each experiment and consisted of direct-acting mutagens for nonactivation assays and mutagens that require metabolic biotransformation in activation assays.

Negative controls consisted of the test material solvent in the overlay agar together with the other essential components. The negative control plate for each strain gave a reference point to which the test data was compared. The positive control assay was conducted to demonstrate that the test systems were functional with known mutagens.

The following normal range of revertants for solvent controls are generally considered acceptable.

TA-1535:	8-30
TA-1537:	4-30
TA-98:	20-75
TA-100:	80-250

D. Evaluation Criteria for Ames Assay

Because the procedures to be used to evaluate the mutagenicity of the test material are semiquantitative, the criteria to be used to determine positive effects are inherently subjective and are based primarily on a historical data base. Most data sets will be evaluated using the following criteria.

1. Strains TA-1535 and TA-1537

If the solvent control value is within the normal range, a test material that produces a positive dose response over three concentrations with the highest increase equal to three times the solvent control value will be considered to be mutagenic.

2. Strains TA-98 and TA-100

If the solvent control value is within the normal range, a test material that produces a positive dose response over three concentrations with the highest increase equal to twice the solvent control value for TA-98 and TA-100 will be considered to be mutagenic.

3. Pattern

Because TA-1535 and TA-100 are both derived from the same parental strain (G-46), to some extent there is a built-in redundancy in the microbial assay. In general, the two strains of a set respond to the same mutagen and such a pattern is sought. Generally, if a strain responds to a mutagen in nonactivation tests, it will do so in activation tests.

4. Reproducibility

If a test material produces a response in a single test that cannot be reproduced in additional runs, the initial positive test data lose significance.

The preceding criteria are not absolute, and other extenuating factors may enter into a final evaluation decision. However, these criteria will be applied to the majority of situations and are presented to aid those individuals not familar with this procedure. As the data base is increased, the criteria for evaluation can be more firmly established.

E. Relation Between Mutagenicity and Carcinogenicity

It must be emphasized that the Ames *Salmonella*/Microsome Plate Assay is not a definitive test for chemical carcinogens. It is recognized, however, that correlative and functional relations have been demonstrated between these two endpoints. The results of comparative tests on 300 chemicals by McCann *et al*.[4] show an extremely good correlation between results of microbial mutagenesis tests and *in vivo* rodent carcinogenesis assays.

All evaluations and interpretation of the data to be presented in the final report will be based only on the demonstration, or lack, of mutagenic activity.

F. Criteria for Ranking Samples in the Ames Assay

The goal of EPA Level 1 Ames testing is to rank source streams by relative degree of genetic toxicity (mutagenicity). Samples are first identified as mutagenic or nonmutagenic by the criteria in Section D above and then ranked using the mutagenicity categories presented in the table below. The lowest concentration giving a positive response in any strain, with or without metabolic activation, is identified as the minimum effective concentration (MEC) for that sample. The mutagenicity of the sample is evaluated as high (H), moderate (M), low (L), or nondetectable (ND) according to the evaluation criteria developed in the Level 1 manual[1] and summarized below. Samples with no detectable activity at the maximum applicable dose (MAD) are ranked nondetectable (ND).

Ames Assay Mutagenicity Ranking Criteria[1]

Mutagenic Activity	Solids (MEC in μg/plate)	Liquids[a] (MEC in μl/plate)
High (H)	<50	<2
Moderate (M)	50-500	2-20
Low (L)	500-5000	20-200
Not Detectable (ND)	>5000	>200

[a] Concentration of organic extracts is based upon organic content (μg organics per plate) and not volume (μl extract per plate) of sample tested.

VIII. REFERENCES

1. Brusick, D.J., et al.: IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests. EPA Contract No. 68-02-2681, Technical Directive No. 501, Litton Bionetics, Inc., Kensington, MD, September 1980, 177 pp. In press.

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GENETICS ASSAY NO.: 5880
LBI SAFETY NO.: 7164

CYTOTOXIC EVALUATION OF A81-05-030-650 (EA-1 XAD EXTRACT) IN THE RODENT CELL (CHO) CLONAL TOXICITY ASSAY

FINAL REPORT

SUBMITTED TO:

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MOUNTAIN VIEW, CALIFORNIA 94042

SUBMITTED BY:

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LBI PROJECT NO. 22064

REPORT DATE: NOVEMBER 1981

Litton BIONETICS

PREFACE

This assay conforms to the standard EPA Level 1 procedure for the Chinese hamster ovary cell (CHO) clonal toxicity assay as described in "IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests" (1). The data were evaluated and formatted as recommended in "Level 1 Biological Testing Assessment and Data Formatting" (2).

The CHO clonal toxicity assay has been shown to be a sensitive method for detecting cytotoxic activity for a variety of chemicals representing various chemical classes (3). This assay is one of several recommended by EPA to identify, categorize and rank the pollutant potential of influent and effluent streams from industrial and energy-producing processes. This assay has been well validated with a wide range of positive and negative control chemicals and complex environmental samples.

All procedures and documents pertaining to the receipt, storage, preparation, testing and evaluation of the test material shall conform to Litton Bionetics, Inc. standard operating procedures and the Good Laboratory Practices Regulations of 1979. Deviations from standard procedure shall be fully documented and noted in the report.

All test and control results in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Molecular Toxicology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20895. Copies of raw data will be supplied to the sponsor upon request.

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I. ASSAY SUMMARY

A. SPONSOR: Acurex Corporation

B. MATERIAL (TEST COMPOUND): GENETICS ASSAY NUMBER: 5879

1. Identification: A81-05-030-650 (EA-1 XAD Extract)
2. Date Received: August 26, 1981
3. Physical Description: Clear, amber-brown liquid

C. TYPE OF ASSAY: Rodent Cell (CHO) Clonal Toxicity Assay

D. ASSAY DESIGN NUMBER: 442

E. STUDY DATES:

1. Initiation: September 23, 1981
2. Completion: November 24, 1981

F. SUPERVISORY PERSONNEL:

1. Study Director: Brian C. Myhr, Ph.D.
2. Laboratory Supervisor: Robert Young, M.S.

G. EVALUATION:

The test material was assayed, as a DMSO extract, over the concentration range of 0.01 µl/ml to 20 µl/ml. A very sharp increase in toxicity occurred in the vicinity of 0.1 µl/ml in the course of two trials. The EC_{50} was estimated to be 0.1 µl/ml, which was equivalent to 1.8 µg of organics/ml. Although the exact position of the EC_{50} appeared to vary between the two trials, the values remained in the high (H) toxicity category defined by the evaluation criteria for the IERL-EPA Level 1 CHO Clonal Toxicity Assay[1].

Submitted by:

Study Director

Brian Myhr 11/24/81

Brian Myhr, Ph.D. Date
Associate Director,
Department of Molecular
Toxicology

Reviewed by:

11/24/81

David J. Brusick, Ph.D. Date
Director,
Department of Molecular
Toxicology

II. OBJECTIVE

The objective of this study was to determine and rank the cytotoxicity of A81-05-030-650 (EA-1 XAD extract) to cultured Chinese hamster cells (CHO-K1 cell line). The measure of cytotoxicity was the reduction in colony-forming ability after a 24-hour exposure to the test material. After a period of recovery and growth, the number of colonies that developed in the treated cultures was compared to the colony number in unexposed vehicle control cultures. The concentration of test material that reduced the colony number by 50% was estimated graphically and referred to as the EC50 value. Standard EPA Level 1 toxicity evaluation criteria for the CHO clonal toxicity assay were used to rank the toxicity potential of the test material.

III. TEST MATERIAL

A. Description

The test material was received as a clear, amber-brown solution in methylene chloride. The sample contained 75.0 milligrams of organic material in 0.7 ml of methylene chloride. No information on the sampling parameters (such as the equivalent volume of stack gas represented by the sample) was provided.

B. Handling and Preparation

The test material was received at LBI on August 26, 1981. The sample was assigned LBI safety number 7163 and LBI assay number 5879. The sample was stored at +4°C in the dark.

Pretest sample preparation consisted of solvent-exchanging the sample into dimethylsulfoxide (DMSO). The sample was transferred with methylene chloride rinses into a graduated conical tube. The methylene chloride was gradually evaporated (50°C under a stream of nitrogen) and DMSO was sequentially added. The sample was brought to volume in 4.1 ml of DMSO, giving a sample concentration of 18.3 mg organics per ml DMSO. The sample was then transferred to a glass vial and sealed with a teflon-coated rubber septum.

A total volume of 0.45 ml of test sample was used in the CHO assay. The maximum concentration of 20 µl/ml was obtained by adding 0.12 ml of sample to 5.88 ml of F12 medium; this resulted in 2% (v/v) DMSO in the medium and effectively limited the concentration of test material that could be assayed. Only two plates were exposed to the high dose in order to conserve sample. Another 0.12 ml aliquot of sample was used to prepare the 10 µl/ml test concentration. An additional 0.21 ml of test sample was used to prepare a series of dilutions in DMSO from which 1:100 dilutions into growth medium were performed to obtain the lower assayed concentrations. Thus, except for the 20 µl/ml test concentration, the final DMSO concentration was constant at 1% (v/v).

IV. MATERIALS

A. Indicator Cells

The indicator cells for this study were Chinese hamster CHO-K1 cells (ATCC No. CCL 61) obtained from Flow Laboratories, Inc., Rockville, MD. This cell type was derived from ovarian tissue and has spontaneously transformed to a stable, hypodiploid line of rounded, fibroblastic cells with unlimited growth potential. Monolayer cultures have a fast doubling time of 11 to 14 hours, and untreated cells can normally be cloned with an efficiency of 80% or greater. Laboratory stock are maintained by routine serial subpassage. Cells are cultivated in Ham's F-12 nutrient medium at 37°C in 5 percent CO_2 with saturated humidity. Stocks are continually observed macroscopically and microscopically for possible microbial contamination. Laboratory cultures are periodically checked by culturing and staining methods for the absence of mycoplasma contamination. Laboratory cultures are discarded every three months and new cultures started from mycoplasma-free, long-term frozen cultures.

B. Media

The CHO-K1 cell line has an absolute requirement for proline and therefore must be maintained in culture medium containing sufficient amounts of this amino acid. Ham's F12 medium, which contains 3×10^{-4} M L-proline was used, supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 0.9 µl/ml of amphotericin B. A 10X formulation of Ham's F10 is available commercially and was used for the testing of aqueous test samples in order to avoid the dilution of medium components. This medium contains 1×10^{-4} L-proline and was supplemented in the same manner as F12, except that kanamycin at 40 µg/ml is included for additional protection against bacterial contamination. Both media formulations support the growth and cloning of CHO cells equally well.

C. Controls

The negative control consisted of three untreated cultures carried through the same experimental time period as the treated cells. Since the test material was tested as a solution in an organic vehicle (DMSO) and was diluted into the medium to provide each test concentration, two sets of vehicle control cultures containing the organic solvent at 1% and 2% by volume were prepared in triplicate.

The average number of colonies in the negative control established the cloning efficiency of the CHO cells used in the assay, and the appropriate vehicle controls provided the reference points for determining the effects of different concentrations of the test material on cell survival.

V. EXPERIMENTAL DESIGN

A. Dose Selection

Unless the approximate toxicity is already known or the sample size is limiting, the following dose ranges are usually tested for different sample forms. Aqueous samples, suspensions, or slurries are tested from 600 µl/ml to 3 µl/ml, usually in six dose steps. Eight doses are often used when the amount of test sample is limited to provide a more precise description of toxicity in the event of sharp dose-response curves. Dry, particulate material is dissolved or suspended in DMSO, diluted into growth medium, and tested at six dose levels from 1000 µg/ml to 3 µg/ml. Samples that are solvent-exchanged into DMSO are tested from 20 µl/ml (2% DMSO in growth medium) to 0.2 µl/ml, also in six dose steps. A second dose study is performed with an adjusted dose range if the EC50 was not located properly in the initial test. However, EC50 values greater than 1000 µg/ml for particulate material, 600 µl/ml for aqueous samples, or 20 µl/ml for organic solutions will not be determined.

This sample, A81-05-030-650 (EA-1 XAD extract), was tested at eight dose levels. The concentrations started with the maximum applicable dose (MAD) of 20 µl/ml and included 10, 6, 3, 1, 0.6, 0.3, and 0.1 µl/ml. The corresponding concentration of organics at the MAD level was 366 µg/ml; the lower doses were equivalent to 183, 109.8, 54.9, 18.3, 11.0, 5.5, and 1.8 µg of organics/ml.

B. Clonal Toxicity Assay

Cells from monolayer stock cultures in logarithmic growth phase were trypsinized with 0.1% trypsin plus 0.01% versene for 4 minutes and the density of the resulting cell suspension determined by hemocytometer. A number of 60-mm culture dishes were then seeded with 200 cells and 4 ml of culture medium per dish. The cultures were incubated for approximately 6 hours at 37°C in a humidified atmosphere containing 5% CO_2 to allow attachment of the cells. The 6-hour attachment period was used in order to avoid cell division and the subsequent formation of two-cell colonies prior to treatment.

The medium was aspirated from the cultures and 4 ml medium containing the test material applied. Three cultures were exposed to each test concentration. After an exposure time of 24 hours at 37°C, the medium was removed by aspiration and each culture washed three times with approximately 4 ml aliquots of Dulbecco's phosphate buffered saline (pre-warmed to 37°C). Fresh culture medium (5 ml) was placed in each dish and incubation at 37°C is continued for an additional 6 days to allow colony development.

The test material caused a color change in the culture medium, the pH of the medium containing the high dose would be determined at the time of treatment. The pH at the lowest dose that results in a slight color change would also recorded. At the end of the treatment period, the pH values of the discarded media from the two described treatments would be recorded again. No sample related pH effects were noted.

After the incubation period, the medium was drained from the cultures and the surviving colonies fixed with 100% ethanol and stained with Giemsa. Colonies were counted by eye; tiny colonies of approximately 50 cells or less were arbitrarily excluded from the counts.

VI. ASSAY ACCEPTANCE CRITERIA

The assay is considered acceptable for evaluation of the test results if the following criteria are met:

- The average cloning efficiency of the CHO-K1 cells in the negative controls is 70% or greater, but not exceeding 115%.
- The distribution of colonies in the treated cultures is generally uniform over the surface of the culture dish.
- The data points for each test concentration critical to the location of the EC50 are the averages of at least two treated cultures.
- A sufficient number of test concentrations are available to clearly locate the EC50 within a toxicity region as defined under Assay Evaluation Criteria.
- If the EC50 value is greater than 1000 µg/ml, 600 µliters of aqueous sample/ml, or 20 µliters of nonaqueous sample/ml, the plotted curve does not exceed 110% of the negative control.

VII. RESULTS

A. Interpretation

The test material, A81-05-030-650 (EA-1 XAD extract), was highly toxic to the CHO cells in the first trial. As shown in Table 1, only the low dose of 0.1 µl/ml resulted in any surviving colonies (15.6% survival). These results indicated that the EC_{50} was less than 0.1 µl/ml or 1.8 µg of organics/ml. Since EC_{50} values below 10 µg/ml are in the high toxicity region defined for the IERL-EPA CHO clonal toxicity bioassay[1], the test material was clearly categorized as having high (H) toxicity to CHO cells.

A very small amount of the test material was available for a second trial, so an attempt was made to locate the EC_{50}. Concentrations from 0.01 µl/ml to 0.3 µl/ml were tested, and the results are presented in Table 2. Also, the relative survivals were plotted along with the results from the first trial in Figure 1. A comparison of the two trials indicated that the EC_{50} had shifted to a value greater than 0.1 µl/ml in the second trial. The survival curve was very sharp. It is not unusual for sharp curves to shift between trials, so the results were analyzed by considering a curve that appeared to be intermediate between the two tirals (dashed line in Figure 1). Thus, a sharp break in survival was estimated to be centered, on the average, at an EC_{50} of 0.1 µl/ml (1.8 µg organics/ml). Individual trials might yield values ranging from 0.06 to 0.16 µl/ml (1.1 to 2.9 µg orgaincs/ml).

The cells used for the assay were in logarithmic growth phase and were greater than 98 percent viable for both trials. About 73 percent of the seeded cells in trial 1 and 77 percent of the seeded cells in trial 2 were able to form colonies as shown by the negative control results. Colony growth was normal and well distributed on the culture dishes. The combined results were considered to achieve assay acceptance criteria and provided confidence in the assumption that the recorded data represented typical responses to the test material.

B. Tables and Figures

This report is based on the data provided in Tables 1 and 2 and Figure 1.

TABLE 1
RODENT CELL (CHO) CLONAL TOXICITY ASSAY

Sample Identity: A81-05-030-650 (EA-1 XAD Extract)

Description of Sample: Clear, amber-brown liquid

LBI Assay No.: 5879

Date Received: August 26, 1981

Test Date: September 28, 1981 (Trial 1)

Vehicle: DMSO

Cell Type: CHO-K1

Cells Seeded per Dish: 200

EC50 Value: <1.8 μg/ml

Toxicity Classification: High

pH Alterations: None

Comments on Treatment: Sample prepared in DMSO at a concentration of 18.3 μg organics/μl

COLONY COUNTS

Sample	Applied Concentration μl/ml	Dish #1	Dish #2	Dish #3	Average Count	Relative Survival[a] %	Cloning Efficiency %
NC[b]	---	146	152	140	146.0	---	73.0
VC, 1%[c]	10	143	125	155	141.0	100.0	70.5
VC, 2%	20	112	110	121	114.3	100.0	57.2
TEST	0.1	21	26	19	22.0	15.6	
TEST	0.3	0	0	0	0	0	
TEST	0.6	0	0	0	0	0	
TEST	1.0	0	0	0	0	0	
TEST	3.0	0	0	0	0	0	
TEST	6.0	0	0	0	0	0	
TEST	10.0	0	0	0	0	0	
TEST	20.0	0	0	S[d]	0	0	

---

[a] Relative to 2% VC for 20 μl/ml treatment and to 1% VC for other treatments.
[b] NC = Negative Control, F_{12} medium.
[c] VC = Vehicle Control, percent DMSO given.
[d] Only two plates dosed to conserve limited test material.

TABLE 2
RODENT CELL (CHO) CLONAL TOXICITY ASSAY

Sample Identity: A81-05-030-650 (EA-1 XAD Extract)

Description of Sample: Clear, amber brown liquid

LBI Assay No.: 5879

Date Received: August 26, 1981

Test Date: November 17, 1981 (Trial 2)

Vehicle: F12 Medium

Cell Type: CHO-K1

Cells Seeded per Dish: 200

Estimated EC50 Value: 0.1 µl/ml (1.8 µg organics /ml)

Toxicity Classification: High

pH Alterations: None

Comments on Treatment: Sample prepared in DMSO in DMSO at a concentration of 18.3 µg organics/µl

CLONAL TOXICITY DATA

Sample	Applied Concentration µl/ml	Dish #1	Dish #2	Dish #3	Average Count	Relative Survival[a] %	Cloning Efficiency %
NC[b]	---	141	155	167	154.3	100.0	77.2
TEST	0.01	154	155	144	151.0	97.9	
TEST	0.02	130	137	138	135.0	87.5	
TEST	0.04	140	135	139	138.0	89.4	
TEST	0.06	137	136	133	135.3	87.7	
TEST	0.08	141	130	131	134.0	86.8	
TEST	0.1	134	125	127	128.7	83.4	
TEST	0.3	10	6	8	8.0	5.2	

[a] Relative to F12 negative control for all treatments.
[b] NC = Negative Control, F12 medium.

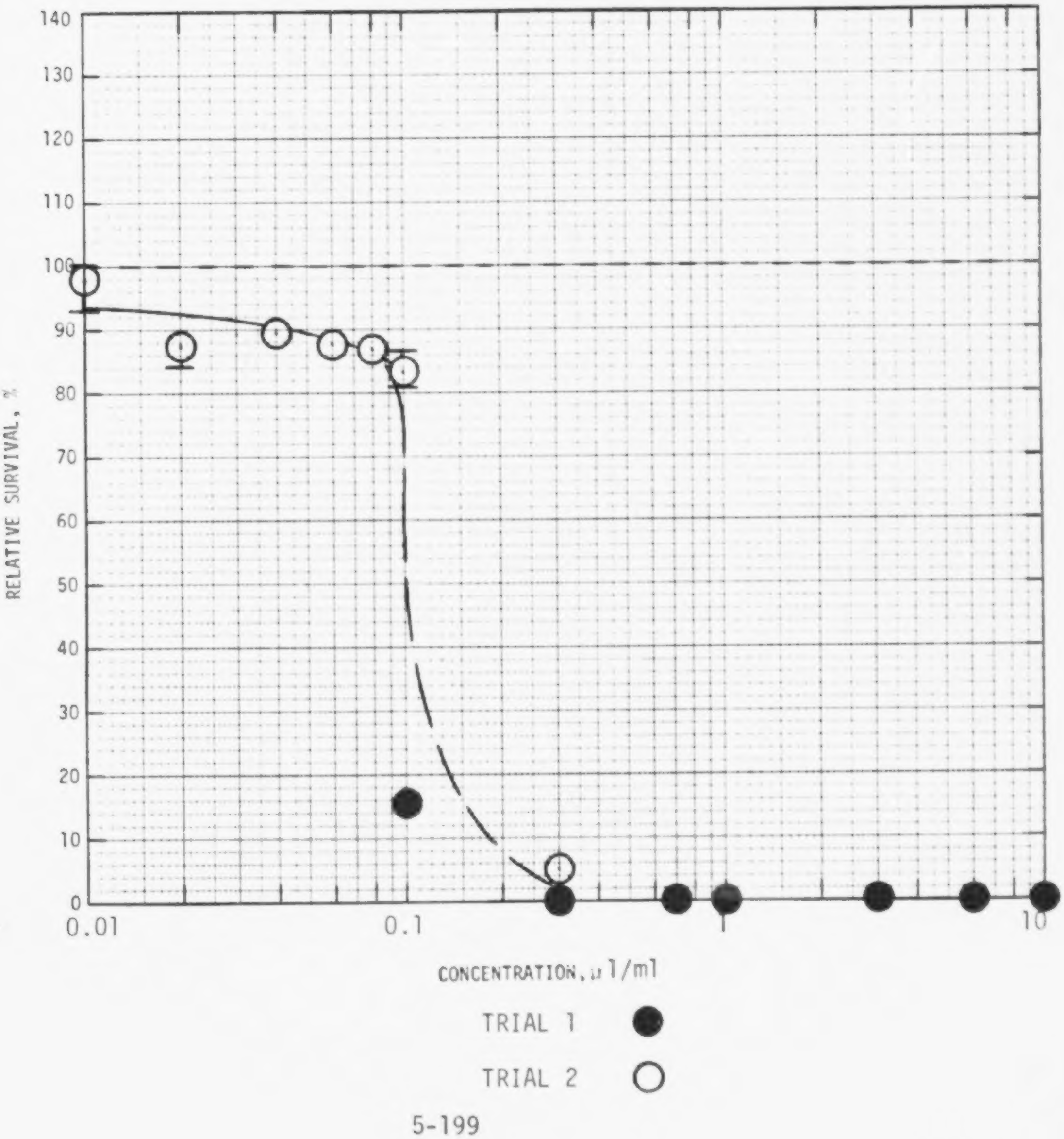
FIGURE 1

RODENT CELL (CHO) CLONAL TOXICITY ASSAY

EC_{50} DETERMINATION

A81-05-030-650

(EA-1 XAD EXTRACT)



VIII. ASSAY EVALUATION CRITERIA

The EC50 value represents the concentrations of test material that reduces the colony-forming ability of CHO cells to 50% of the vehicle or negative control value. EC50 values are determined graphically by fitting a curve by eye through relative survival data plotted as a function of the logarithm of the applied concentration. Each data point normally represents the average of three culture dishes. In order to indicate the variability of the data, the high and low colony counts for each concentration are used to calculate the relative survivals, and the range is shown by a bar at the position of the plotted average. If no bar is shown, the variability was within the size of the symbol. Statistical analysis is unnecessary in most cases for evaluation.

The toxicity of the test material is evaluated as high, moderate, low, or nondetectable according to the range of EC50 values defined in the following table.

Toxicity[a]	Solids (EC_{50} in μg/ml)	Aqueous Liquids (EC_{50} in μl/ml)	Nonaqueous Liquids[b] (EC_{50} in μl/ml)
High	<10	<6	<0.2
Moderate	10 to 100	6 to 60	0.2-2
Low	100 to 1000	60 to 600	2-20
Not Detectable	>1000	>600	>20

[a] Evaluation criteria formulated by Litton Bionetics, Inc. for IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests.

[b] Criteria for nonaqueous liquids are tentative and under evaluation. If the organic or solids content is known, the sample is evaluated under the solids criteria.

Another evaluation scheme is proposed for extracts obtained from SASS train gas volumes. The proportion of the total gas volume corresponding to the volume of extract used in the bioassay is calculated and expressed as L/ml of culture medium (or DSCF/ml of culture medium). A criterion of 1000 L/ml is set as the limit for nondetectable toxicity. This gas volume corresponds to the average volume breathed by humans over a 2-hour period. The subsequent toxicity ranges are defined by 10-fold dilution steps to conform to standard procedure. The toxicity ranges are defined in the following table for liter and dry standard cubic feet units:

Toxicity	EC_{50} In Liters/ml (L/ml)	EC_{50} In Dry Standard Cubic Feet/ml (DSCF/ml)
High	<10	<0.35 DSCF
Moderate	10-100	0.35-3.5
Low	100-1000	3.5-35
Nondetectable	>1000	>35

IX. REFERENCES

1. Brusick, D.J., *et al.*: *IERL-RTP Procedures Manual*: *Level 1 Environmental Assessment Biological Tests*. EPA Contract No. 68-02-2681, Technical Directive No. 501, Litton Bionetics, Inc., Kensington, MD, September 1980, 177 pp. In press.

2. Brusick, D.J.: *Level 1 Bioassay Assessment and Data Formatting*. EPA-600/7-80-079, Litton Bionetics, Inc., Kensington, MD, April 1980, 100 pp.

3. Brusick, D.J. and Young, R.R.: *Level 1 Bioassay Sensitivity*. EPA-600/7-81-135, Litton Bionetics, Inc., Kensington, MD, August 1981, pp 52.

GENETICS ASSAY NO.: 5886
LBI SAFETY NO.: 7170

MUTAGENICITY EVALUATION OF A81-05-030-662 (EA-1 FLYASH) IN THE EPA LEVEL 1 AMES SALMONELLA/MICROSOME PLATE TEST

FINAL REPORT

SUBMITTED TO:

ACUREX CORPORATION
485 CLYDE AVENUE
MOUNTAIN VIEW, CALIFORNIA 94042

SUBMITTED BY:

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5516 NICHOLSON LANE
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LBI PROJECT NO.: 22064

REPORT DATE: NOVEMBER 1981

Litton BIONETICS

PREFACE

This assay conforms to the standard EPA Level 1 procedure for the Ames Salmonella/microsome mutagenesis assay as described in "IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests"[1]. The data were evaluated and formatted as recommended in "Level 1 Biological Testing Assessment and Data Formatting"[2].

The Ames Salmonella/microsome mutagenesis assay has been shown to be a sensitive method for detecting mutagenic activity for a variety of chemicals representing various chemical classes[3]. This assay is one of several recommended by EPA to identify, categorize and rank the pollutant potential of influent and effluent streams from industrial and energy-producing processes. This assay has been well validated with a wide range of positive and negative control chemicals and complex environmental samples.

All procedures and documents pertaining to the receipt, storage, preparation, testing and evaluation of the test material shall conform to Litton Bionetics, Inc. standard operating procedures and the Good Laboratory Practices Regulations of 1979. Deviations from standard procedure shall be fully documented and noted in the report.

All test and control results in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Molecular Toxicology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20895. Copies of raw data will be supplied to the sponsor upon request.

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I. ASSAY SUMMARY

A. Sponsor: Acurex Corporation

B. Material (Test Compound): Genetics Assay Number: 5886

1. Identification: A81-05-030-662 (EA-1 Flyash)
2. Date Received: August 26, 1981
3. Physical Description: Black and gray particles

C. Type of Assay: EPA Level 1 Ames Salmonella/Microsome Plate Test

D. Assay Design Number: 401 (EPA Level 1)

E. Study Dates:

1. Initiation: September 23, 1981
2. Completion: September 28, 1981

F. Supervisory Personnel:

A. Study Director: D.R. Jagannath, Ph.D.

G. Evaluation:

The test material, A81-05-030-662 (EA-1 flyash), was evaluated for its genetic activity in the EPA Level 1 Ames *Salmonella* assay directly and in the presence of a metabolic activation system. The test material was preincubated in dimethylsulfoxide at 37°C overnight in a rotary shaker before testing. Testing was conducted over a concentration range of 0.05 mg/plate to 5.0 mg/plate. The test was performed in duplicate under nonactivation and activation test conditions with strains TA-1535, TA-1537, TA-98, and TA-100.

The results of the nonactivation and activation assays were negative. Based on the mutagenicity results, the mutagenic activity of the test material was ranked as nondetectable (ND) according to the EPA Level 1 evaluation criteria for the Ames Assay[1].

Submitted by:

Study Director

D.R. Jagannath 11/24/81

D.R. Jagannath, Ph.D. Date
Section Chief,
Submammalian Genetics,
Department of Molecular
Toxicology

Reviewed by:

11/24/81

David J. Brusick, Ph.D. Date
Director,
Department of Molecular
Toxicology

II. OBJECTIVE

The objective of this study was to determine the genetic activity of A81-05-030-662 (EA-1 flyash) in the Salmonella/ microsome assay with and without the addition of mammalian metabolic activation preparations. The genetic activity of a sample is measured in these assays by its ability to revert the Salmonella indicator strains from histidine dependence to histidine independence. The degree of genetic activity of a sample is reflected in the number of revertants that are observed on the histidine-free medium.

III. TEST MATERIAL

A. Description

The test material was received as black and gray particles (15 gm) and was used without further preparation. No information on actual particle size distribution or on sampling parameters was received.

B. Handling and Preparation

The test material was received at LBI on August 26, 1981. The sample was assigned LBI safety number 7170 and LBI assay number 5886. The sample was stored at +4°C in the dark.

A total of 313.08 mg of test material was weighed and suspended in 3.13 ml of dimethylsulfoxide. The sample formed an opaque suspension that settled upon standing. The suspension was incubated at 37°C on a shaker overnight to help leach material out of the particulates. Serial dilutions were made in DMSO such that 50 µl aliquots of each dilution give the desired concentration. The suspension was well mixed when aliquots were removed for dosing.

IV. MATERIALS

A. Indicator Microorganisms

The *Salmonella typhimurium* strains used in this assay were obtained from Dr. Bruce Ames, University of California at Berkeley.[4-8] The following four strains were used.

Strain Designation	Gene Affected	Additional Mutations			Mutation Type Detected
		Repair	LPS	R Factor	
TA-1535	*his* G	Δ *uvr* B	*rfa*	-	Base-pair substitution
TA-1537	*his* C	Δ *uvr* B	*rfa*	-	Frameshift
TA-98	*his* D	Δ *uvr* B	*rfa*	pKM101	Frameshift
TA-100	*his* G	Δ *uvr* B	*rfa*	pKM101	Base-pair substitution

All the above strains have, in addition to the mutation in the histidine operon, mutation (*rfa*-) that leads to defective lipopolysaccharide coat, a deletion that covers genes involved in the synthesis of vitamin biotin (*bio*-) and in the repair of ultraviolet (uv) - induced DNA damage (*uvr*B-). The *rfa*- mutation makes the strains more permeable to many large molecules. The *uvr*B- mutation decreases repair of some types of chemically or physically damaged DNA and thereby enhances the strain's sensitivity to some mutagenic agents. The resistant transfer factor plasmid (R factor) pKM101 in TA-98 and TA-100 is believed to cause an increase in error-prone DNA repair that leads to many more mutations for a given dose of most mutagens.[8] In addition, plasmid pKM101 confers resistance to the antibiotic ampicillin, which is a convenient marker to detect the presence of plasmid in the cells.

All indicator strains are kept at 4°C on minimal medium plates supplemented with a trace of biotin and an excess of histidine. In addition, the plates with plasmid-carrying strains contain ampicillin (25 µg/ml) to ensure stable maintenance of plasmid pKM101. New stock culture plates are made as often as necessary from the frozen master cultures or from single colony reisolates that were checked for their genotypic characteristics (*his*, *rfa* *uvrB*, *bio*) and for the presence of plasmid. For each experiment, an inoculum from the stock culture plates is grown overnight at 37°C in nutrient broth (Oxoid CM67) and used.

B. Media

The bacterial strains were cultured in Oxoid Media #2 (Nutrient Broth). The selective medium was Vogen Bonner Medium E with 2% glucose.[10] The

overlay agar consisted of 0.6% purified agar with 0.05 mM histidine, 0.05 mM biotin and 0.1M NaCl according to the methods of Ames *et al*.[9]

C. Activation System

1. S9 Homogenate

A 9,000 x *g* supernatant prepared from Sprague-Dawley adult male rat liver induced by Aroclor 1254 (Ames *et al*.[9]) was purchased commercially and used in these assays.

2. S9 Mix

S9 mix used in these assays consisted of the following components:

Components	Concentration per Milliliter S9 Mix
NADP (sodium salt)	4 μmoles
D-glucose-6-phosphate	5 μmoles
$MgCl_2$	8 μmoles
KCl	33 μmoles
Sodium phosphate buffer pH 7.4	100 μmoles
Organ homogenate from rat liver (S9 fraction)	100 μliters

V. EXPERIMENTAL DESIGN

A. Dosage Selection

Test strategy and dose selection depend upon sample type and sample availability. The Level 1 manual[1] recommends solids to be initially tested at the maximum applicable dose (MAD) of 5 mg per plate and at lower concentrations of 2.5, 1, 0.5, 0.1 and 0.05 mg per plate. Liquids are tested initially at the MAD of 200 µl per plate, and at lower concentrations of 100, 50 and 10 µl per plate. Samples are retested over a narrower range of concentrations with strains showing positive results initially. Alternate dose are employed if sample size is limiting or at the direction of the sponsor.

Doses selected to test this sample covered the recommended dose range for solids. The highest dose was at the MAD level of 5.0 mg per plate and included five lower dose levels of 2.5, 1, 0.5, 0.1 and 0.05 mg per plate.

B. Mutagenicity Testing

The procedure used was based on the paper published by Ames et. al.[9] and was performed as follows:

1. Nonactivation Assay

To a sterile 13 x 100 mm test tube placed in a 43°C water bath the following was added in order:

- 2.00 ml of 0.6% agar containing 0.05 mM histidine and 0.05 mM biotin.
- 0.05 ml of a suspension of the test chemical to give the appropriate dose.
- 0.1 ml to 0.2 ml of indicator organism(s).
- 0.50 ml of 0.2M phosphate buffer, pH 7.4.

This mixture was swirled gently and then poured onto minimal agar plates (see IV B, Media). After the top agar had set, the plates were incubated at 37°C for approximately 2 days. The number of his+ revertant colonies growing on the plates were counted with an automatic colony counter and recorded.

2. Activation Assay

The activation assay was run concurrently with the nonactivation assay. The only difference was the addition of 0.5 ml of S9 mix (see IV C, Activation System) to the tubes in place of 0.5 ml of phosphate buffer which was added in nonactivation assays. All other details were similar to the procedure for nonactivation assays.

A detailed flow diagram for the plate incorporation assay is provided in Figure 1.

C. Control Compounds

A negative control consisting of the solvent used for the test material was also assayed concurrently with the test material. For negative controls, step 'b' of Nonactivation Assays was replaced by 0.05 ml of the solvent. The negative controls were employed for each indicator strain and were performed in the absence and presence of S9 mix. The solvent used to prepare the stock solution of the test material is given in the Results section of this report. All dilutions of the test material were made using this solvent. The amount of solvent used was equal to the maximum volume used to give the appropriate test dose.

Specific positive control compounds known to revert each strain were also used and assayed concurrently with the test material. The concentrations and specificities of these compounds to specific strains are given in the following table:

Assay	Chemical	Solvent	Concentration per plate (μg)	Salmonella Strains
Nonactivation	Sodium azide	Water	10.0	TA-1535, TA-100
	2-Nitrofluorene (NF)	Dimethylsulfoxide	10.0	TA-98
	9-aminoacridine (9AA)	Ethanol	50.0	TA-1537
Activation	2-anthramine (ANTH)	Dimethylsulfoxide	2.5	For all strains

D. Recording and Presenting Data

The number of colonies on each plate were counted and recorded on printed forms. These raw data were analyzed in a computer program and reported on a printout. The results are presented as revertants per plate for each indicator strain employed in the assay. The positive and solvent controls are provided as reference points.

AMES ASSAY [PLATE INCORPORATION METHOD]

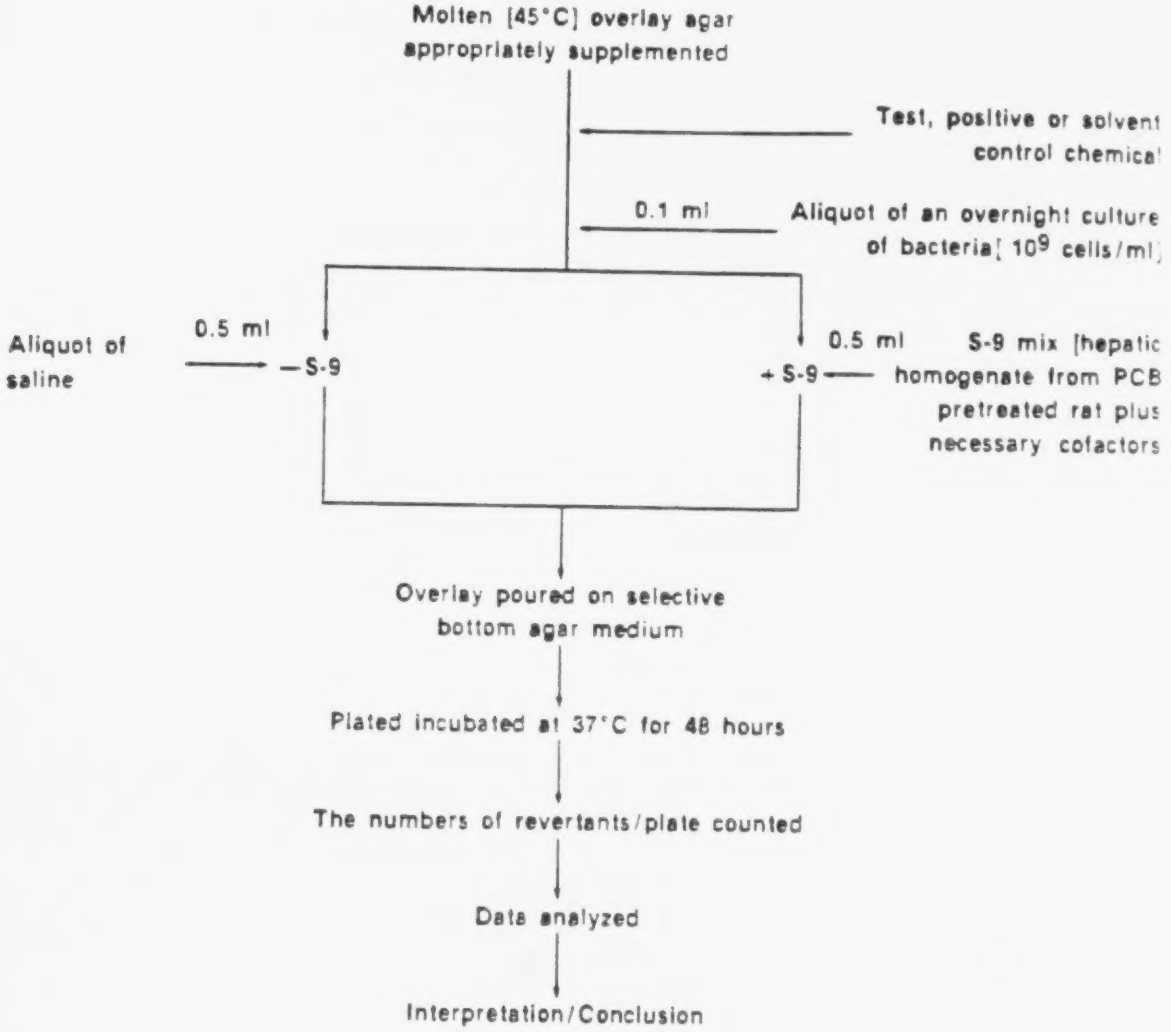
Molten [45°C] overlay agar
appropriately supplemented
Test, positive or solvent
control chemica
0.1 ml
Aliquot of an overnight culture
of bacteria[10^9 cells/ml]
Aliquot of
saline
0.5 ml
—S-9
0.5 ml
+S-9
S-9 mix [hepatic
homogenate from PCB
pretreated rat plus
necessary cofactors
Overlay poured on selective
bottom agar medium
Plated incubated at 37°C for 48 hours
The numbers of revertants/plate counted
Data analyzed
Interpretation/Conclusion

Figure 1 AMES SALMONELLA/MICROSOME MUTAGENESIS ASSAY

VI. RESULTS

A. Interpretations

The test material, A81-05-030-662 (EA-1 flyash), was dissolved in DMSO at a stock concentration of 100 mg/ml and leached overnight on a shaker at 37°C. Additional dilutions were prepared in DMSO for testing. The maximum test level was 5.0 mg/plate.

Reverse mutation was measured in strains TA-1535, TA-1537, TA-98 and TA-100. The test was conducted in duplicate both with and without rat liver S9 mix for metabolic activation.

There was no mutagenic activity associated with the test material treatment and the sample was considered nonmutagenic and non toxic. The sample was ranked as having nondetectable (ND) mutagenic activity using the IERL-EPA Level 1 evaluation criteria for the Ames Assay[1].

Solvent control and positive control values were within acceptable ranges. These results achieved assay acceptance criteria and provided confidence in the assumptions that the recorded data represented typical responses to the test material.

B. Tables

This report is based on the data provided in Table 1.

```
 RESULTS                                               TABLE 1
--------------------------------

A.    NAME OR CODE DESIGNATION OF THE TEST COMPOUND:  A81-05-030-662 (EA-1 FLYASH)
B.    SOLVENT:  DMSO
C.    TEST INITIATION DATES:  09/24/81
D.    TEST COMPLETION DATE: 09/28/81
E.    S-9 LOT#: S-9-11
NOTE:   CONCENTRATIONS ARE GIVEN IN MILLIGRAMS    PER  PLATE
                                          REVERTANTS  PER  PLATE
                                       ---------------------------------------------------------------------
TEST                 SPECIES TISSUE    TA-1535          TA-1537          TA-98            TA-100
----                 ------- ------ ---------------- ---------------- ---------------- ----------------
                                         1    2    3     1    2    3     1    2    3     1    2    3
NONACTIVATION
-------------


SOLVENT CONTROL       ---      ---      12   17          9   12         46   38         132  106
POSITIVE CONTROL**    ---      ---    1076  961        621  628        745  811        1308 1359

TEST COMPOUND
       0.050    MG    ---      ---      18   12          7   12         47   27         145  130
       0.100    MG    ---      ---      13   14          9   11         32   46         126  137
       0.500    MG    ---      ---      14   15          8   11         44   33         156  166
       1.000    MG    ---      ---      15   15          8   12         46   33         133  165
       2.500    MG    ---      ---      14   12         12   14         47   29         149  168
       5.000    MG    ---      ---      14   15         14    6         41   53         143  157

ACTIVATION
----------


SOLVENT CONTROL       RAT     LIVER     17   11         13    8         45   34         101  123
POSITIVE CONTROL***   RAT     LIVER    308  254        339  372       1562 1600        2065 1832

TEST COMPOUND
       0.050    MG    RAT     LIVER      7   11         11    8         51   49         138  115
       0.100    MG    RAT     LIVER      7    3         12   13         44   57         118  127
       0.500    MG    RAT     LIVER     13    9         13   14         38   47         109  126
       1.000    MG    RAT     LIVER     10    7         10   12         43   48         121  128
       2.500    MG    RAT     LIVER     14    7         13   11         48   41         128  125
       5.000    MG    RAT     LIVER     10    6         15   20         53   55         143  129
---------------------------------------------------------------------------------------------------------

**                                                         ***
   TA-1535   SODIUM AZIDE                  10 UG/PLATE        TA-1535   2-ANTHRAMINE   2.5 UG/PLATE
   TA-1537   9-AMINOACRIDINE               50 UG/PLATE        TA-1537   2-ANTHRAMINE   2.5 UG/PLATE
   TA-98     2-NITROFLUORENE               10 UG/PLATE        TA-98     2-ANTHRAMINE   2.5 UG/PLATE
   TA-100    SODIUM AZIDE                  10 UG/PLATE        TA-100    2-ANTHRAMINE   2.5 UG/PLATE
   SOLVENT   50 UL/PLATE
```

VII. ASSAY ACCEPTANCE AND EVALUATION CRITERIA

Statistical methods are not currently used, and evaluation is based on the criteria included in this protocol.

Plate test data consists of direct revertant colony counts obtained from a set of selective agar plates seeded with populations of mutant cells suspended in a semisolid overlay. Because the test material and the cells are incubated in the overlay for approximately 2 days and a few cell divisions occur during the incubation period, the test is semiquantitative in nature. Although these features of the assay reduce the quantitation of results, they provide certain advantages not contained in a quantitative suspension test:

- The small number of cell divisions permits potential mutagens to act on replication DNA, which is often more sensitive than nonreplicating DNA.
- The combined incubation of the test article and the cells in the overlay permits constant exposure of the indicator cells for approximately 2 days.

A. Surviving Populations

Plate test procedures do not permit exact quantitation of the number of cells surviving chemical treatment. At low concentrations of the test material, the surviving population on the treatment plates is essentially the same as that on the negative control plate. At high concentrations, the surviving population is usually reduced by some fraction. Our protocol will normally employ several doses ranging over two or three log concentrations, the highest of these doses being selected to show slight toxicity as determined by subjective criteria.

B. Dose-Response Phenomena

The demonstration of dose-related increased in mutant counts is an important criterion in establishing metagenicity. A factor that might modify dose-response results for a mutagen would be the selection of doses that are too low (usually mutagenicity and toxicity are related). If the highest dose is far lower than a toxic concentration, no increases may be observed over the dose range selected. Conversely, if the lowest dose employed is highly cytotoxic, the test material may kill any mutants that are induced, and the test material will not appear to be mutagenic.

C. Control Tests

Positive and negative control assays were conducted with each experiment and consisted of direct-acting mutagens for nonactivation assays and mutagens that require metabolic biotransformation in activation assays.

Negative controls consisted of the test material solvent in the overlay agar together with the other essential components. The negative control plate for each strain gave a reference point to which the test data was compared. The positive control assay was conducted to demonstrate that the test systems were functional with known mutagens.

The following normal range of revertants for solvent controls are generally considered acceptable.

TA-1535: 8-30
TA-1537: 4-30
TA-98: 20-75
TA-100: 80-250

D. Evaluation Criteria for Ames Assay

Because the procedures to be used to evaluate the mutagenicity of the test material are semiquantitative, the criteria to be used to determine positive effects are inherently subjective and are based primarily on a historical data base. Most data sets will be evaluated using the following criteria.

1. Strains TA-1535 and TA-1537

If the solvent control value is within the normal range, a test material that produces a positive dose response over three concentrations with the highest increase equal to three times the solvent control value will be considered to be mutagenic.

2. Strains TA-98 and TA-100

If the solvent control value is within the normal range, a test material that produces a positive dose response over three concentrations with the highest increase equal to twice the solvent control value for TA-98 and TA-100 will be considered to be mutagenic.

3. Pattern

Because TA-1535 and TA-100 are both derived from the same parental strain (G-46), to some extent there is a built-in redundancy in the microbial assay. In general, the two strains of a set respond to the same mutagen and such a pattern is sought. Generally, if a strain responds to a mutagen in nonactivation tests, it will do so in activation tests.

4. Reproducibility

If a test material produces a response in a single test that cannot be reproduced in additional runs, the initial positive test data lose significance.

The preceding criteria are not absolute, and other extenuating factors may enter into a final evaluation decision. However, these criteria will be applied to the majority of situations and are presented to aid those individuals not familar with this procedure. As the data base is increased, the criteria for evaluation can be more firmly established.

E. Relation Between Mutagenicity and Carcinogenicity

It must be emphasized that the Ames *Salmonella*/Microsome Plate Assay is not a definitive test for chemical carcinogens. It is recognized, however, that correlative and functional relations have been demonstrated between these two endpoints. The results of comparative tests on 300 chemicals by McCann *et al*.[4] show an extremely good correlation between results of microbial mutagenesis tests and *in vivo* rodent carcinogenesis assays.

All evaluations and interpretation of the data to be presented in the final report will be based only on the demonstration, or lack, of mutagenic activity.

F. Criteria for Ranking Samples in the Ames Assay

The goal of EPA Level 1 Ames testing is to rank source streams by relative degree of genetic toxicity (mutagenicity). Samples are first identified as mutagenic or nonmutagenic by the criteria in Section D above and then ranked using the mutagenicity categories presented in the table below. The lowest concentration giving a positive response in any strain, with or without metabolic activation, is identified as the minimum effective concentration (MEC) for that sample. The mutagenicity of the sample is evaluated as high (H), moderate (M), low (L), or nondetectable (ND) according to the evaluation criteria developed in the Level 1 manual[1] and summarized below. Samples with no detectable activity at the maximum applicable dose (MAD) are ranked nondetectable (ND).

Ames Assay Mutagenicity Ranking Criteria[1]

Mutagenic Activity	Solids (MEC in μg/plate)	Liquids[a] (MEC in μl/plate)
High (H)	<50	<2
Moderate (M)	50-500	2-20
Low (L)	500-5000	20-200
Not Detectable (ND)	>5000	>200

[a] Concentration of organic extracts is based upon organic content (μg organics per plate) and not volume (μl extract per plate) of sample tested.

VIII. REFERENCES

1. Brusick, D.J., et al.: IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests. EPA Contract No. 68-02-2681, Technical Directive No. 501, Litton Bionetics, Inc., Kensington, MD, September 1980, 177 pp. In press.

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GENETICS ASSAY NO.: 5886
LBI SAFETY NO.: 7170

CYTOTOXIC EVALUATION OF A81-05-030-662 (EA-1 FLYASH) IN THE RABBIT ALVEOLAR MACROPHAGE (RAM) CYTOTOXICITY ASSAY

FINAL REPORT

SUBMITTED TO:

ACUREX CORPORATION
485 CLYDE AVENUE
MOUNTAIN VIEW, CALIFORNIA 94042

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20895

LBI PROJECT NO.: 22064

REPORT DATE: NOVEMBER 1981

Litton BIONETICS

PREFACE

This assay conforms to the standard EPA Level 1 procedure for the rabbit alveolar macrophage (RAM) cytotoxicity assay as described in "IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests" (1). The data were evaluated and formatted as recommended in "Level 1 Biological Testing Assessment and Data Formatting" (2).

The RAM cytotoxicity assay has been shown to be a sensitive method for detecting cytotoxic activity for a variety of chemicals representing various chemical classes (3). This assay is one of several recommended by EPA to identify, categorize and rank the pollutant potential of influent and effluent streams from industrial and energy-producing processes. This assay has been well validated with a wide range of positive and negative control chemicals and complex environmental samples.

All procedures and documents pertaining to the receipt, storage, preparation, testing and evaluation of the test material shall conform to Litton Bionetics, Inc. standard operating procedures and the Good Laboratory Practices Regulations of 1979. Deviations from standard procedure shall be fully documented and noted in the report.

All test and control results in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Molecular Toxicology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20895. Copies of raw data will be supplied to the sponsor upon request.

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I. ASSAY SUMMARY

A. SPONSOR: Acurex Corporation

B. MATERIAL (TEST COMPOUND): GENETICS ASSAY NUMBER: 5886

1. Identification: A81-05-030-662 (EA-1 Flyash)
2. Date Received: August 26, 1981
3. Physical Description: Gray, black powder with small chunks

C. TYPE OF ASSAY: Rabbit Alveolar Macrophage (RAM) Cytotoxicity Assay

D. ASSAY DESIGN NUMBER: 443

E. STUDY DATES:

1. Initiation: September 23, 1981
2. Completion: October 23, 1981

F. SUPERVISORY PERSONNEL:

1. Study Director: Brian Myhr, Ph.D.
2. Laboratory Supervisor: Robert Young, M.S.

G. EVALUATION:

The test material, after being ground to a fine powder, caused a dose-related increase in toxicity for applied concentrations greater than 300 μg/ml. The viability index and ATP content were the most sensitive assay parameters and both indicated an EC_{50} near 1000 μg/ml. This result was evaluated as showing low/nondetectable (L/ND) borderline toxicity for the test material in the RAM Cytotoxicity Assay, according to the IERL-EPA Level 1 toxicity categories.

Submitted by:

Study Director

Brian Myhr 11/19/81
Brian Myhr, Ph.D. Date
Associate Director,
Department of Molecular
Toxicology

1/19/81
David J. Brusick, Ph.D. Date
Director,
Department of Molecular
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II. OBJECTIVE

The objective of this study was to determine and rank the cytotoxicity of A81-05-030-662 (EA-1 Flyash) to cultured rabbit alveolar macrophage (RAM) cells. The measure of cytotoxicity was the reduction in cell viability and adenosine triphosphate (ATP) content of the cultures after a 20 hour exposure to the test material. At the conclusion of the exposure period, the number of viable cells and total ATP content in the treated cultures were compared to the corresponding values in unexposed control cultures. The concentration of test material that reduced each experimental parameter by 50% was estimated graphically and referred to as the EC50 value. Standard EPA Level 1 toxicity evaluation criteria for the RAM cytotoxicity assay were used to rank the toxicity potential of the test material based upon the most sensitive parameter.

III. TEST MATERIAL

A. Description

The test material was received as a gray powder containing small, black particles. The total amount of sample supplied was 15 grams. No information on the sampling parameters was provided.

B. Handling and Preparation

The test material was received on August 26, 1981, and was assigned LBI assay number 5886 and LBI safety number 7170. The sample was stored at +4°C in the dark.

Approximately 28 mg of the test material was tested as supplied. Then, on October 1, 1981, the remaining sample was ground in a mortar and pestle to fine gray powder. Approximately 3.0 grams of the ground sample was further pulverized on October 8, 1981, to a very fine, gray powder of which 36 mg was used in the second trial of the assay. For both trials, the test material was suspended in serum-free EMEM culture medium at a concentration of 2000 µg/ml and incubated at 37°C on a roller drum for 8 hours. The original material settled quickly on standing, but the suspension formed from the pulverized powder remained well-dispersed for dilutions. No pH changes were observed. The suspensions were serially diluted with EMEM (serum-free) and applied to the cultures at a maximum concentration of 1000 µg/ml in the presence of 10% serum.

IV. MATERIALS

A. Indicator Cells

Both assay trials employed short-term primary cultures of alveolar macrophage cells obtained by lung lavage of male New Zealand white rabbits (2.0-2.5 kg). The rabbits were maintained on Purina Lab Rabbit Chow 5321 and water *ad libitum* and were examined for the absence of respiratory illnesses prior to use.

B. Media

The cells were maintained and treated in Eagle's Minimum Essential Medium (EMEM) with Earle's salts and supplemented with 10% fetal bovine serum (heat-inactivated), 100 units/ml penicillin, 100 µg/ml streptomycin, 17.6 µg/ml kanamycin, and 0.4 µg/ml amphotericin B.

C. Negative Controls

The negative control for the first trial consisted of six untreated cultures carried through the same experimental time period as the treated cells. Six cultures were used because a large number of cells was obtained by pooling the yield from two rabbits in order to run two concurrent assays. Only one animal was used for the second trial, and the usual three untreated cultures were prepared. The average viability and ATP content of the negative controls provided the reference points for determining the effects of different concentrations of the test material on the assay parameters.

V. EXPERIMENTAL DESIGN

A. Procurement of Cells

The rabbits were sacrificed by injection of Nembutal® (60 mg/ml) into the marginal ear vein, and sterile operating techniques were used to perform a tracheostomy. Prewarmed normal saline (30 ml) was then introduced into the lungs via a catheter and allowed to stand for 15 minutes. This lavage fluid was removed and placed into a 50-ml sterile centrifuge tube on ice. Nine additional lavages were similarly performed and collected, except the saline was removed shortly after its introduction into the lungs. Any lavage fluid containing blood or mucous was discarded. The lavages were centrifuged at 365 x g for 15 minutes and the cells resuspended in cold 0.85% saline. After two washes in saline by centrifugation, the cell pellets were resuspended in cold EMEM containing 20% serum and then combined. A cell count was obtained by hemocytometer and the suspension diluted to between 5×10^5 and 10^6 cells/ml. Viability was determined by trypan blue staining and the cells were not used if less than 95% viable. Also, a differential cell count from Wright-stained smears was performed to verify that the macrophage content was above 90%.

B. Sample Forms

The usual sample form for application to the cells is a suspension of particulate material. Solid samples are ground to fine particles and a weighed portion is suspended in a known volume of EMEM (0% FBS) for about eight hours to help leach any water-soluble material. Finely-divided test material may be suspended directly in culture medium without further grinding. Aqueous liquids, suspensions, or slurries containing less than 0.5% organic solvent are added by volume to culture medium.

Samples supplied as solutions in organic solvents are usually solvent-exchanged into DMSO before testing. Original sample volumes may first be reduced a maximum of 10-fold in a Kuderna-Danish concentrator, and the concentrative factor is used to convert assayed volumes into equivalent original sample volumes in the absence of information about solute concentration. An aliquot of the reduced volume is exchanged into DMSO by repeated, partial evaporation under a stream of nitrogen in a warm water bath (50°C); the evaporated volumes are replaced with equal volumes of DMSO.

Samples adsorbed on XAD-2 resin are extracted with methylene chloride or acetone in a Soxhlet apparatus for 24 hours. The extract is then concentrated and solvent-exchanged into DMSO. Alternatively, acetone extracts can be assayed directly at concentrations up to 2% by volume in the culture medium.

Samples impregnated on fiber glass or teflon filters are repeatedly sonicated in cyclohexane to remove particulates. The resulting cyclohexane particulate suspension is then evaporated to dryness and the particulates resuspended in EMEM culture medium at the desired concentration.

Sponsor-specified handling of sample materials will be followed if the above procedures are not applicable or a specific procedure is desired.

C. Dose Selection

Unless the approximate toxicity is already known or the sample size is limiting, the following usual dose ranges are tested for different sample forms. Dry, particulate material is tested at six dose levels from 1000 µg/ml to 3 µg/ml. Aqueous samples, suspensions, or slurries are tested from 600 µl to 3 µl/ml in six dose steps. Samples that are solvent-exchanged into DMSO are tested from 20 µl/ml (2% DMSO in growth medium) to 0.2 µl/ml, also in six dose steps. A second dose study is performed with an adjusted dose range if the EC50 was not located properly in the initial test. However, EC50 values greater than 1000 µl/ml for particulate material, 600 µl/ml for aqueous samples, or 20 µl/ml for organic solutions will not be determined.

This test material, A81-05-030-662 (EA-1 flyash), was tested as supplied at 6 dose levels, starting at the maximum applicable dose (MAD) of 1000 µg/ml and including 600, 300, 100, 60 and 30 µg/ml. The second trial was performed with only three doses of the finely ground test material: 1000, 600 and 300 µg/ml.

D. Treatment

A series of 25 cm^2 culture flasks were prepared, each containing 2.0 ml of serum-free medium at 37°C and the test material at twice the desired final concentration. Three flasks were prepared for each test concentration. Aliquots of cell suspension (2 ml) were then added; each flask, therefore, contained from 1 to 2 x 10^6 viable cells in a 4-ml volume of media containing 10% serum. The flasks were placed on a rocker platform in a 37°C incubator with a humidified atmosphere containing 5% CO_2. After sitting for about 30 minutes, the flasks were slowly rocked for the remainder of a 20-hour exposure period.

If the test substance causes a color change in the growth medium, the pH is determined in additional treated flasks. After the exposure period, the pH of the medium in the experimental flasks is again recorded.

E. Cell Viability Assay

At the end of the treatment period, the medium containing unattached cells was decanted into a centrifuge tube on ice. The attached cells were rinsed with 1 ml of 0.1% trypsin/0.01% versene and then incubated with 2 ml of the trypsin/versene solution for about 5 minutes at 37°C. The trypsinates and decanted media were combined for each culture to yield a 7-ml cell suspension for subsequent analysis.

A 0.5 ml aliquot of the cell suspension was removed for cell count and viability determination. The aliquot was combined with 1.0 ml of 0.4% trypan blue and counted by hemocytometer about 5 to 15 minutes later.

The total number of cells counted per culture was the sum of the numbers found in five squares for each chamber of the hemocytometer (1 µl total volume). The numbers of live (colorless) and dead (blue) cells were recorded.

F. ATP Assay

ATP was immediately analyzed by extraction of a 0.1-ml sample of cell suspension with 0.9 ml of 90% DMSO. After 2 minutes at room temperature 5.0 ml cold MOPS buffer (0.01 M morpholinopropane sulfonic acid) at pH 7.4 was added and the extract mixed well and placed on ice. Aliquots of 10 µl were injected into a cuvette containing a luciferin-luciferase reaction mixture in a DuPont Model 760 Luminescence Biometer. The Biometer was calibrated daily with standard ATP solutions to provide a direct read-out of the ATP content. Each test sample was assayed at least twice to obtain repeatable readings.

VI. ASSAY ACCEPTANCE CRITERIA

The assay will be considered acceptable for evaluation of the test results if the following criteria are met:

1. The macrophage population is 90% or greater of the total nucleated cells collected by lung lavage.
2. The percent viability of the macrophages used to initiate the assay is 95% or greater.
3. The survival of viable macrophages in the negative control cultures over the 20 hour treatment priod is 70% or greater.
4. A sufficient number of data points (for five test concentrations or less) are available to clearly locate the EC50 of the most sensitive test parameter within a toxicity region as defined under Assay Evaluation Criteria.
5. The data points critical to the location of the EC50 for the most sensitive parameter are the averages of at least two treated cultures.
6. If all the test parameters yield EC50 values greater than 1000 μg/ml, 600 μl/ml for aqueous solutions, or 20 μl/ml for organic solutions, the plotted curves for ATP content and viability index parameters do not exceed 120% of the negative control.

VII. RESULTS

A. Interpretation

The original test material, which consisted of many coarse particles, did not interact appreciably with the macrophages. As shown in Table 1 and Figures 1 and 2, the assay parameters remained near the negative control values for all tested doses up to 1000 µg/ml. However, when the test material was pulverized to a fine powder, a toxic response was observed at concentrations above 300 µg/ml. The results for the fine powder are presented in Table 2 and Figures 3 and 4. Absolute and relative assay parameters are provided in the tables, whereas the relative values are plotted in the Figures to determine EC_{50} positions.

The viability index (which measures cell survival) and the culture ATP content usually tend to parallel each other, and an inspection of the curves in Figures 3 and 4 show this to be the case for the current assay. Both parameters were essentially equally sensitive and indicated an EC_{50} near 1000 µg/ml. This value is on the borderline between the low (L) and nondetectable (ND) toxicity categories defined for the IERL-EPA Level 1 RAM assay[1]. Since the EC_{50} position will shift slightly in either direction with repeated trials, the toxicity was evaluated as low/nondetectable (L/ND) borderline. The results from the second trial were considered more relevant than those from the first trial because the small particle size allowed ingestion by the macrophages.

The percent viability assay parameter was unaffected in both trials. This behavior indicated the cells disrupted soon after ingesting the particles. The decline in cell count was shown by the decreased viability index and was probably responsible for the lack of response for the $ATP/10^6$ cells assay parameter.

The macrophages collected for the assays had normal morphology and appeared to be in a healthy state. The initial viability was approximately 99% and the survival of viable cells in the negative controls was also about 99% in both trials. The average cellular ATP content ($ATP/10^6$ total cells) for the negative controls was within the historical range for acceptable cultures. These results achieved the assay acceptance criteria and provided confidence in the assumption that the collected data represented typical responses to the test material.

B. Tables and Figures

This report is based on the data provided in Tables 1 and 2 and Figures 1 to 4.

TABLE 1
RABBIT ALVEOLAR MACROPHAGE (RAM) CYTOTOXICITY ASSAY DATA

LBI Assay No.: 5886 (Trial I, Unground sample)

Test Material Identity: A81-05-030-662 (EA-1 Flyash)

Test Date: September 23, 1981

Vehicle: EMEM

Initial Cell Viability: 98.8%

Viable Macrophage Seeded/Flask: 2.0 x 10^6 cells/flask

Macrophage Population Percentage: >90.0%

Survival of Negative Control Macrophage Over Treatment Time: 99.1%

Sample	Concentration[a] µg/ml	Average Values per Culture Flask			ATP Per 10^6 Cells 10^8 fg	Viability %	Expressed as Percent of Negative Control			
		Viable Cells 10^6 Units	Total Cells 10^6 Units	ATP 10^8 fg[b]			Viability	Viability Index	ATP	ATP Per 10^6 Cells
NC[c]	---	2.14	2.16	66.4	30.7	99.1	100.0	100.0	100.0	100.0
TEST	30	2.06	2.08	65.1	31.3	99.0	99.9	96.3	98.0	102.0
TEST	60	2.21	2.25	67.2	29.9	98.2	99.1	103.3	101.2	97.4
TEST	100	2.16	2.20	66.8	30.4	98.2	99.1	100.9	100.6	99.0
TEST	300	1.82	1.84	64.3	34.9	98.9	99.8	85.0	96.8	113.7
TEST	600	2.02	2.03	62.6	30.8	99.5	100.4	94.4	94.3	100.3
TEST	1000	1.95	2.02	60.5	30.0	96.5	97.4	91.1	91.1	97.7

[d]EC50 VALUES: µg/ml:	>1000	>1000	>1000	>1000

Toxicity Classification: Nondetectable

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[a] pH change in culture medium: None observed

[b] fg = Femtogram (10^{-15} gram).

[c] NC = Negative Control, EMEM culture medium.

[d] Determined from data plots in Figures 1 and 2.

TABLE 2

RABBIT ALVEOLAR MACROPHAGE (RAM) CYTOTOXICITY ASSAY DATA

LBI Assay No.: 5886 (Trial II, Ground sample)

Test Material Identity: A81-05-030-662 (EA-1 Flyash)

Test Date: October 22, 1981

Vehicle: EMEM

Initial Cell Viability: 94.4%

Viable Macrophage Seeded/Flask: 1.03 x 10^6 cells/flask

Macrophage Population Percentage: >90%

Survival of Negative Control Macrophage Over Treatment Time: 98.9%

Sample	Concentration[a] µg/ml	Average Values per Culture Flask: Viable Cells 10^6 Units	Average Values per Culture Flask: Total Cells 10^6 Units	Average Values per Culture Flask: ATP 10^8 fg[b]	ATP Per 10^6 Cells 10^8 fg	Viability %	Expressed as Percent of Negative Control: Viability	Expressed as Percent of Negative Control: Viability Index	Expressed as Percent of Negative Control: ATP	Expressed as Percent of Negative Control: ATP Per 10^6 Cells
NC[c]	---	0.89	0.90	26.1	29.0	98.9	100.0	100.0	100.0	100.0
TEST	300	0.83	0.86	23.1	26.9	96.5	97.6	93.3	88.5	92.8
TEST	600	0.72	0.74	19.9	26.9	97.3	98.4	80.9	76.2	92.8
TEST	1000	0.42	0.44	14.2	32.3	95.5	96.6	47.2	54.4	111.4

[d]EC50 VALUES: µg/ml: Viability >1000; Viability Index ~1000; ATP ~1000; ATP Per 10^6 Cells >1000

Toxicity Classification: Low/Nondetectable Borderline

---

[a] pH change in culture medium: None observed

[b] fg = Femtogram (10^{-15} gram).

[c] NC = Negative Control, EMEM culture medium.

[d] Determined from data plots in Figures 1 and 2.

FIGURE 1

EC50 DETERMINATION FOR

PERCENT VIABILITY (O) AND VIABILITY INDEX (●)

A81-05-030-662

(EA-1 FLYASH)

TRIAL 1

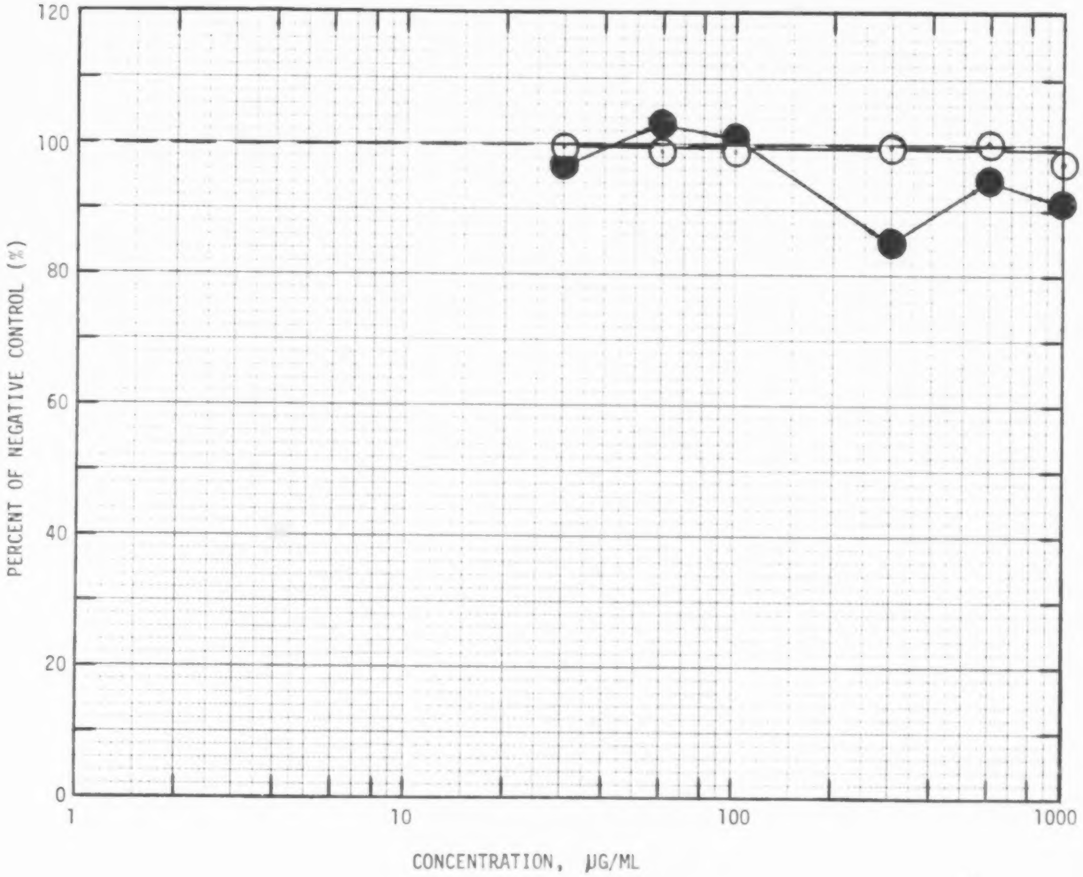


FIGURE 2

EC50 DETERMINATION FOR

ATP/FLASK (O) AND ATP/10^6 CELLS (●)

A81-05-030-662

(EA-1 FLYASH)

TRIAL 1

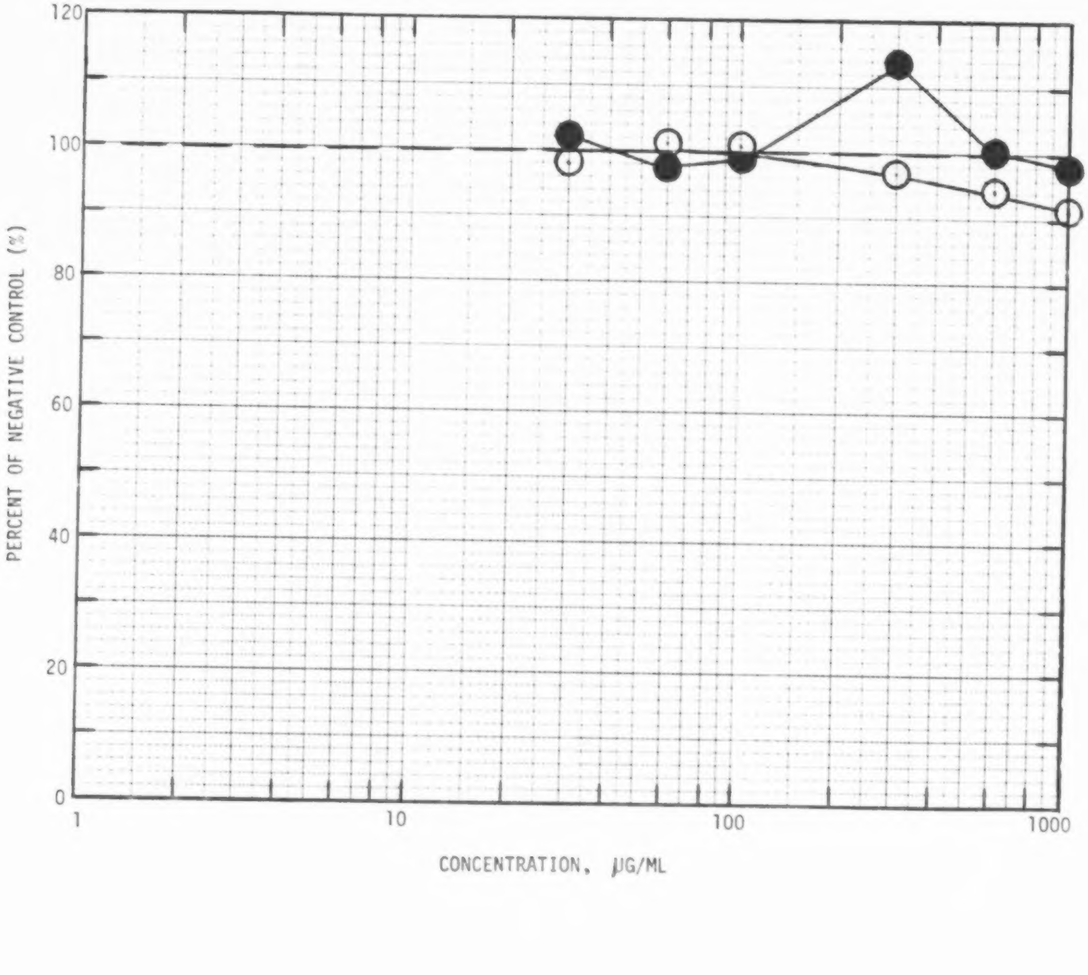


FIGURE 3

EC50 DETERMINATION FOR

PERCENT VIABILITY (O) AND VIABILITY INDEX (●)

A81-05-030-662

(EA-1 FLYASH)

TRIAL 2

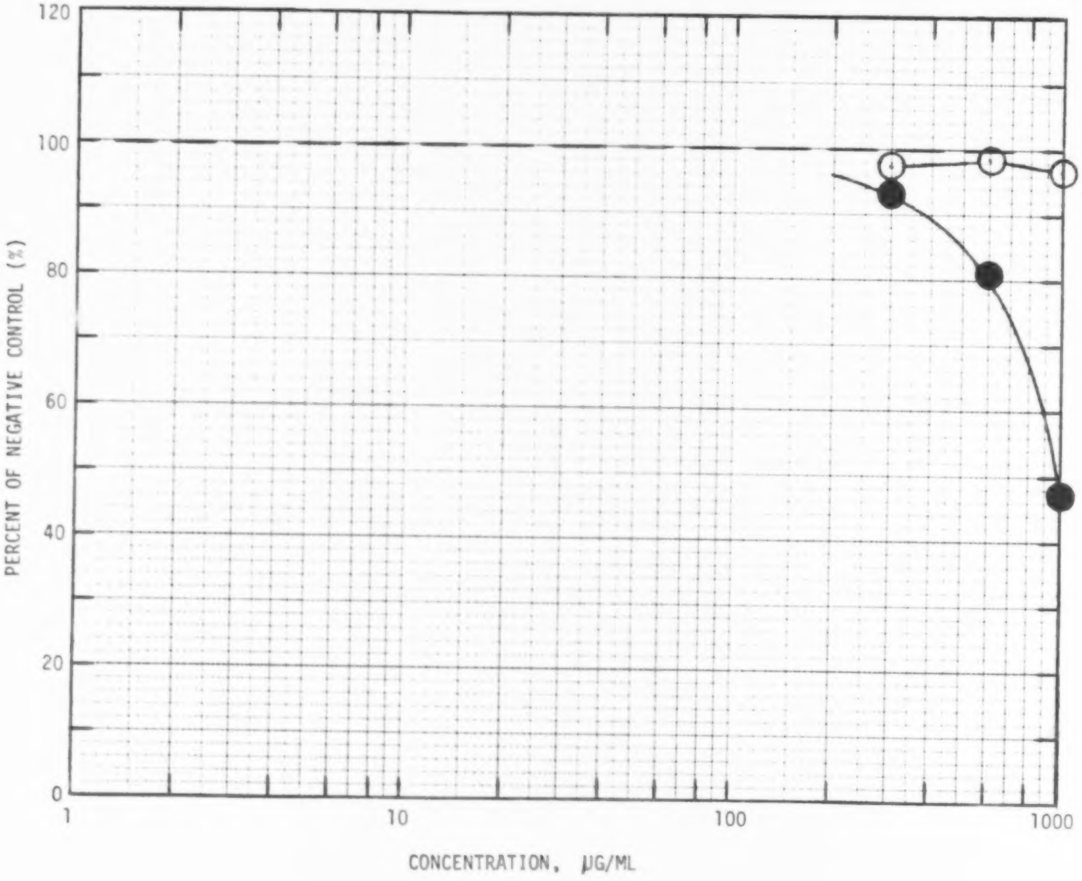


FIGURE 4

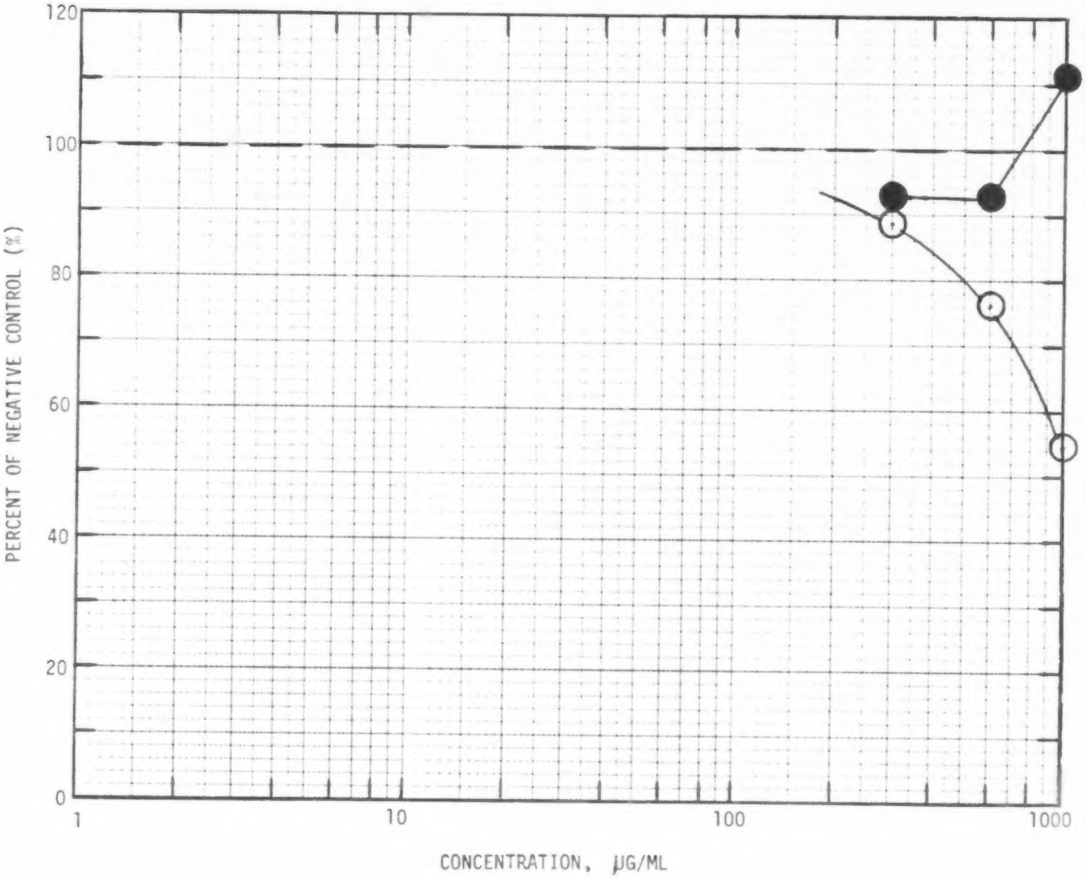
EC50 DETERMINATION FOR

ATP/FLASK (O) AND ATP/10^6 CELLS (●)

A81-05-030-662

(EA-1 FLYASH)

TRIAL 2



VIII. ASSAY EVALUATION CRITERIA

The EC50 value represents the concentration of test material that reduces the most sensitive parameter of the RAM assay to 50% of the vehicle or negative control value. EC50 values are determined graphically by fitting a curve by eye through relative toxicity data plotted as a function of the logarithm of the applied concentration. Each data point normally represents the average of three culture dishes. Statistical analysis is unnecessary in most cases for evaluation.

The toxicity of the test material is evaluated as high, moderate, low, or nondetectable according to the range of EC50 values defined in the following table.

Toxicity[a]	Solids (EC_{50} in µg/ml)	Aqueous Liquids (EC_{50} in µl/ml)	Nonaqueous Liquids[b] (EC_{50} in µl/ml)
High	<10	<6	<0.2
Moderate	10 to 100	6 to 60	0.2-2
Low	100 to 1000	60 to 600	2-20
Not Detectable	>1000	>600	>20

[a] Evaluation criteria formulated by Litton Bionetics, Inc. for IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests[1].

[b] Criteria for nonaqueous liquids are tentative and under evaluation. If the organic or solid content is known, the solid evaluation criteria are applied.

Another evaluation scheme is proposed for extracts obtained from SASS train gas volumes. The proportion of the total gas volume corresponding to the volume of extract used in the bioassay is calculated and expressed as L/ml of culture medium (or DSCF/ml of culture medium). A criterion of 1000 L/ml is set as the limit for nondetectable toxicity. This gas volume corresponds to the average volume breathed by humans over a 2-hour period. The subsequent toxicity ranges are defined by 10-fold dilution steps to conform to standard procedure. The toxicity ranges are defined in the following table for liter and dry standard cubic feet units:

Toxicity	EC_{50} In Liters/ml (L/ml)	EC_{50} In Dry Standard Cubic Feet/ml (DSCF/ml)
High	<10	<0.35 DSCF
Moderate	10-100	0.35-3.5
Low	100-1000	3.5-35
Nondetectable	>1000	>35

IX. REFERENCES

1. Brusick, D.J., et al.: IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests. EPA Contract No. 68-02-2681, Technical Directive No. 501, Litton Bionetics, Inc., Kensington, MD, September 1980, 177 pp. In press.

2. Brusick, D.J.: Level 1 Bioassay Assessment and Data Formatting. EPA-600/7-80-079, Litton Bionetics, Inc., Kensington, MD, April 1980, 100 pp.

3. Brusick, D.J. and Young, R.R.: Level 1 Bioassay Sensitivity. EPA-600/7-81-135, Litton Bionetics, Inc., Kensington, MD, August 1981, pp. 52.

GENETICS ASSAY NO.: 5886
LBI SAFETY NO.: 7170

TOXIC EVALUATION OF A81-05-030-662 (EA-1 FLYASH) IN THE EPA LEVEL 1 ACUTE IN VIVO RODENT TOXICITY ASSAY

FINAL REPORT

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LBI PROJECT NO.: 22064

REPORT DATE: NOVEMBER 1981

BIONETICS

PREFACE

This assay conforms to the standard EPA Level 1 procedure for the acute *in vivo* toxicity test in rodents as described in "IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests"[1]. The data were evaluated and formatted as recommended in "Level 1 Biological Testing Assessment and Data Formatting"[2]. The organisms used in this assay were male and female weanling mice as recommended by the Level 1 Manual.[1]

The advantages of *in vivo* toxicity assays are embodied mainly in the fact that the toxicological assessment is performed in whole animals. There is a significant background of test data on a wide range of toxicants for the rodent systems, thus supplying needed information for the reliable interpretation of results with complex effluents[3]. The main disadvantage of an acute rodent toxicity study is a possibly unsatisfactory prediction of toxicity induced by long-term/ low-level exposures. An additional consideration is the need for multi-gram quantities of test material which may prohibit testing where small amounts of sample are available, such as from source streams containing gaseous and particulate material.

Since the major objective of the Level 1 biological testing procedures is to identify toxicological problems at minimal cost, a two-step approach was developed for the initial acute *in vivo* toxicological evaluation of unknown compounds. The first step *is based* on the quantal (all-or-none) response of dosing animals only at the maximum applicable dose. If no animals die in the quantal test, further *in vivo* testing is not initiated and the sample toxicity is categorized as *not detectable*. If any animals die in the quantal screening, a multiple dose quantitative test is initiated to determine the dose that kills 50 percent of the animals (LD_{50}). The toxicity potential of the test material is then ranked using standard EPA Level 1 toxicity evaluation criteria for the acute *in vivo* rodent toxicity assay[1].

All procedures and documents pertaining to the receipt, storage, preparation, testing and evaluation of the test material shall conform to Litton Bionetics, Inc. standard operating procedures and the Good Laboratory Practices Regulations of 1979. Deviations from standard procedure shall be fully documented and noted in the report.

All test and control results in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Molecular Toxicology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20795. Copies of raw data will be supplied to the sponsor upon request.

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I. ASSAY SUMMARY

A. SPONSOR: Acurex Corporation

B. MATERIAL (TEST COMPOUND): GENETICS ASSAY NO.: 5886

1. Identification: A81-05-030-662 (EA-1 Flyash)
2. Date Received: August 26, 1981
3. Physical Description: Gray and white powder with small black particles.

C. TYPE OF ASSAY: EPA Level 1 Rodent Quantal Toxicity Assay

D. STUDY DATES:

A. Initiation: October 5, 1981

B. Completion: October 23, 1981

E. SUPERVISORY PERSONNEL:

A. Study Director: David J. Brusick, Ph.D.

B. Senior Technician: Joan McGowan

F. EVALUATION:

The test substance, A81-05-030-662 (EA-1 Flyash), was not lethal or toxic to weanling mice following an oral gavage dose of 5 gm/kg body weight. There were no unusual findings upon necropsy that would suggest test substance related toxicity. The test sample response was evaluated as being in the nondetectable (ND) range as defined for the IERL-EPA Level 1 Rodent Quantal Toxicity Assay[1].

Submitted by:

David J. Brusick, Ph.D.
Director
Department of Molecular
Toxicology

11/20/81
Date

II. OBJECTIVES

The objective of this assay was to evaluate the acute toxicity of A81-05-030-662 (EA-1 flyash) when administered by oral gavage to male and female weanling mice. Test strategy involved initial testing of the sample at the maximum applicable dose in the quantal assay. If lethality was observed in the quantal assay, additional testing would be initiated at lower doses to identify the LD_{50}.

The assay consisted of recording any lethality and toxic signs that occurred initially and over a 14-day period following a single treatment. Additional information was collected from necropsy observations on animals that died during the course of the experiment or were killed at the end of the 14-day observation period.

III. TEST MATERIAL

A. Description

The test material, A81-05-030-662 (EA-1 flyash), was received as a gray and white powder containing small, black particles. The amount of sample supplied was 15.0063 grams. No information on the sampling parameters was provided.

B. Handling and Preparation

The test material was received at LBI on August 26, 1981. The sample was assigned LBI safety number 7170 and LBI assay number 5886. The sample was stored at +4°C in the dark.

On October 1, 1981, the test material was ground in a mortar and pestle to a fine, gray powder. The primary dosing suspension was prepared 24 hours in advance to permit water soluble materials in the flyash to leach into the water at room temperature. A total of 1716.83 mg of test material was suspended in 17.07 ml of sterile distilled water giving a stock concentration of 101 mg/ml. This suspension would not pass freely through a 24G gavage needle so it was discarded. On October 8, 1981, approximately 3.0 gm of the previously ground sample was pulverized a second time in a mortar and pestle. The suspension prepared 24 hours in advance of dosing, passed through the gavage needle without difficulty. A total of 1815.5 mg of test material was suspended in 12.0 ml of sterile water giving a stock concentration of 151 mg/ml.

IV. MATERIALS

A. Test Organisms

The test organisms for this study were weanling Charles River CD-1 mice. Weanlings were used because they are likely to be more sensitive to toxic effects of test materials than adult mice. In addition, significantly less test material is required for dosing.

Eight nursing female Charles River CD-1 mice with ten pups each (5 male and 5 female) were obtained from Charles River Breeding Laboratories, Inc., Wilmington, MA on September 30, 1981. The birth date of the pups was September 13, 1981. The animals were quarantined for 5 days upon receipt. The litters were individually housed on Ab-sorb-dri bedding in polycarbonate cages and were cared for according to Litton Bionetics, Inc., Department of Molecular Toxicology and LAMS Standard Operating Procedures. Purina certified laboratory chow and water (pH 2.5) were provided *ad libitum*. The pups were maintained with mothers until weaned. The animals were identified by eartags and cage cards and were released for study on October 9, 1981.

V. EXPERIMENTAL DESIGN

A. Quantal Test

Ten male and ten female weanling CD-1 mice were used in the initial quantal screening test. The pups appeared to be in good health with no physical or behavioral problems noted. Pups that were selected were of similar size. The pups were 26 days old at the time of dosing.

Prior to dosing, each animal was individually weighed and the mean weight calculated for each sex. The volume of test material to be administered was based on the mean weight if all animals were within plus or minus 15 percent of the average for the sex. If any animals were outside that range, they were then excluded from the average, a new mean calculated for the remaining animals and individual dosing volumes calculated for each outlying animals.

The test material was administered by gavage to the pups at the rate of 5 gm/kg. The average weight of the males was 15.1 gm and that of the females was 13.3 gm. All animals were within ±15 percent of the average for the sex. The test material, suspended at the concentration of 151 mg per ml, was applied to the animals in two equal doses that totaled 0.50 ml for the males and 0.44 ml for the females.

Immediately following administration of the test substance and during the first day, observations of the frequency and severity of all toxic signs or pharmacological effects (Table 1) were recorded. Particular attention was paid to time of onset and disappearance of signs. Observations were made and recorded on all animals through a 14-day period. At termination of the observation period, all surviving animals were weighed, killed, and then gross necropsies performed. Necropsies were also performed on all animals that died during the course of this study.

B. Quantitative Test

Since no animals died during the preliminary quantal screening test, the quantitative test to determine the LD_{50} was unneccessary.

TABLE 1. DEFINITION OF PHARMACOLOGICAL TOXIC SIGNS

Organ System	Observation and Examination	Common Signs of Toxicity
CNS and somatomotor	Behavior	Change in attitude to observer, unusual vocalization, restlessness, sedation
	Movements	Twitch, tremor, ataxia, catatonia, paralysis, convulsion, forced movements
	Reactivity to various stimuli	Irritability, passivity, anaesthesis, hyperaesthesis
	Cerebral and spinal reflexes	Sluggishness, absence
	Muscle tone	Rigidity, flaccidity
Autonomic nervous system	Pupil size	Myosis, mydriasis
	Secretion	Salivation, lacrimation
Respiratory	Nostrils	Discharge
	Character and rate of breathing	Bradypnoea, dyspnoea, Cheyne-Stokes breathing, Kussmaul breathing
Cardiovascular	Palpation of cardiac region	Thrill, bradycardia, arrhythmia, stronger or weaker beat
Gastrointestinal	Events	Diarrhea, constipation,
	Abdominal shape	Flatulence, contraction
	Feces consistency and color	Unformed, black or clay colored
	Vulva, mammary glands	Swelling
	Penis	Prolapse
	Perianal region	Soiled
Skin and fur	Color, turgor, integrity	Reddening, flaccid skinfold, eruptions, piloerection
Mucous membranes	Conjunctiva, mouth	Discharge, congestion, hemorrhage cyanosis, jaundice
Eye	Eyeball	Exophthalmus, nystagmus
	Transparency	Opacities
Others	Rectal or paw skin	Subnormal, increased temperature
	General Condition	Abnormal posture, emaciation

VI. RESULTS

A. Interpretation

The test material, A81-05-030-662 (EA-1 flyash), was tested and evaluated in the EPA Level 1 Acute In Vivo Rodent Toxicity Assay. The first phase of testing was the quantal toxicity test in which 10 male and 10 female weanling CD-1 mice were exposed to an oral dose of the test material. This dose was at the maximum applicable dose (MAD) of 5 gm/kg as recommended by the EPA Level 1 procedures manual[1].

All twenty animals survived the exposure with no evidence of any compound-related behavioral or toxic signs (see Table 1 for definitions). The only visable sign related to test material dosing was staining of the muzzle noted in some animals immediately after dosing. Both male and female mice showed good weight gains during the 14-day observation period. At the end of the observation period, the mice were sacrificed and necropsied. Gross necropsy showed no evidence of compound-related lesions. The results of the study are summarized in Table 2.

The test material was evaluated as having nondetectable (ND) toxicity at the MAD of 5 gm/kg in the acute in vivo rodent toxicity assay. No deaths, toxic signs or gross lesions at necropsy were noted. Because no toxic effects were observed at the MAD, the quantitative study (LD_{50} determination) was unnecessary.

B. Tables

This report is based on the data provided in Table 2.

TABLE 2
QUANTAL TOXICITY DATA WTH WEANLING MICE

Quantal Toxicity: Weanling CD-1 mice
Sponsor: Acurex Corporation
Test Article: A81-05-030-662 (EA-1 flyash)
Description: Gray and white powder with black particles. The test material was ground to a fine, gray powder that was used to prepare the dosing suspension.
Vehicle: Sterile, deionized water
Study Dates: October 8, 1981 to October 23, 1981
Animals: Charles River CD-1 mice, P.O. 106949
Dose: 5 gm/kg administered by oral gavage

Animal No.	Initial Weight gm	Final Weight gm	Visible Toxic Signs[a]	Gross Necropsy Findings
Males				
9022	14.8	23.4	NTS[b]	NSL[c]
9023	16.0	23.9	NTS	Large intestine filled with yellow fluid
9024	15.6	24.4	NTS	NSL
9025	14.7	24.5	NTS	NSL
9026	14.0	20.4	Scruffy after dosing	White mucous plug in urinary bladder
9027	13.9	24.6	NTS	NSL
9028	15.0	27.1	NTS	White mucous plug in urinary bladder
9029	15.4	23.1	NTS	Hard lymph node in mammary tissue in lower right intestinal area
9030	15.7	25.1	NTS	NSL
9031	15.4	25.4	NTS	NSL

Mean Body Weight: Initial = 15.1 ± 0.7 gm (Standard Deviation)
Final = 24.2 ± 1.7 gm (Standard Deviation)

---

[a] Animals observed over 14 days.
[b] NTS = No Toxic Signs.
[c] NSL = No Significant Lesions

Note: Staining of the muzzle from the test material was noted in some animals immediately after dosing.

TABLE 2 (Continued)

QUANTAL TOXICITY DATA WTH WEANLING MICE

Animal No.	Initial Weight gm	Final Weight gm	Visible Toxic Signs[a]	Gross Necropsy Findings
Females				
9032	14.7	19.3	NTS[b]	NSL[c]
9033	13.8	20.4	NTS	NSL
9034	14.2	23.3	NTS	NSL
9035	13.7	19.8	NTS	NSL
9036	13.4	19.6	NTS	NSL
9037	12.1	18.5	NTS	NSL
9038	12.7	19.5	NTS	One mesenteric lymph node slightly enlarged
9039	12.0	17.4	NTS	NSL
9040	12.6	18.2	NTS	NSL
9041	13.3	21.4	NTS	Mesenteric lymph nodes slightly enlarged.

Mean Body Weight:

Initial = 13.3 ± 0.9 gm (Standard Deviation)
Final = 19.7 ± 1.7 gm (Standard Deviation)

---

[a] Animals observed over 14 days.
[b] NTS = No Toxic Signs.
[c] NSL = No Significant Lesions

Note: Staining of the muzzle from the test material was noted in some animals immediately after dosing.

VII. EVALUATION CRITERIA

If no mortality occurs in the quantal study, no further studies will be performed with the test substance and the LD_{50} should be reported as greater than 5 ml/kg or 5 g/kg. The test material is then ranked as having nondectable toxicity (ND) at the maximum applicable dose (MAD). Effluent samples which produce harmful effects *in vivo* and do not result in deaths will be noted in the results summary. Such observations are difficult to quantitate but provide insight into the sublethal effects of a sample on rodents. Further investigations may be recommended from observations of nonlethal toxic effects.

If a single animal in the quantal study dies in the 14-day observation period, a quantitative study will be performed. An LD_{50} will be calculated by the method of Litchfield and Wilcoxin[4]. If the data are not suitable for calculation of a precise LD_{50}, i.e., total mortality occurs for the lowest dose, an estimate of the LD_{50} could be made or the LD_{50} could be expressed as 0.05 ml/kg or 0.05 g/kg or less. Occasionally, it may be necessary to use a different series of dosages in a repeat study to accurately locate the LD_{50}. The calculated LD_{50} value is used to rank the toxicity of the test material according to the dose ranges presented in Table 3.

Frequent observations are also made and recorded on all animals through the 14-day period. As in the quantal phase, no attempt is made to quantitate or rank observations. The average animal body weight of each group is determined initially and at the termination of the experiment. The average weights and the weights as fractions of the control are reported for each dose level. Necropsy observations are recorded and reported.

TABLE 3

ACUTE *IN VIVO* RODENT TOXICITY ASSAY EVALUATION CRITERIA

Toxicity[a]	Solids (LD_{50} in g/kg)	Liquids (LD_{50} in ml/kg)
High	<0.05	<0.05
Moderate	0.05 to 0.5	0.05 to 0.5
Low	0.5 to 5	0.5 to 5
Not Detectable	>5	>5

[a] Evaluation criteria formulated by Litton Bionetics, Inc. for *IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests.*[1]

VIII. REFERENCES

1. Brusick, D.J., et al.: IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests. EPA Contract No. 68-02-2681, Technical Directive No. 501, Litton Bionetics, Inc., Kensington, MD, September 1980, 177 pp., in press.

2. Brusick, D.J.: Level 1 Bioassay Assessment and Data Formatting. EPA-600/7-80-079, Litton Bionetics, Inc., Kensington, MD, April 1980, 100 pp.

3. Brusick, D.J. and Young, R.R.: Level 1 Bioassay Sensitivity. EPA 600/7-81-135 Litton Bionetics, Inc., Kensington, MD, August 1981, 52 pp.

4. Litchfield, J.T. and Wilcoxin, F.: "A Simplified Method of Evaluation Dose-Effect Experiments." J. Pharmac. Exp. Ther., Vol. 96, 1949, pp. 99-113.

GENETICS ASSAY NO.: 5883
LBI SAFETY NO.: 7167

MUTAGENICITY EVALUATION OF
A81-05-030-672
(EA-2 10+3)
IN THE
EPA LEVEL 1
AMES SALMONELLA/MICROSOME
PLATE TEST

FINAL REPORT

SUBMITTED TO:

ACUREX CORPORATION
485 CLYDE AVENUE
MOUNTAIN VIEW, CALIFORNIA 94042

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20895

LBI PROJECT NO.: 22064

REPORT DATE: NOVEMBER 1981

Litton BIONETICS

PREFACE

This assay conforms to the standard EPA Level 1 procedure for the Ames *Salmonella*/microsome mutagenesis assay as described in "IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests"[1]. The data were evaluated and formatted as recommended in "Level 1 Biological Testing Assessment and Data Formatting"[2].

The Ames *Salmonella*/microsome mutagenesis assay has been shown to be a sensitive method for detecting mutagenic activity for a variety of chemicals representing various chemical classes[3]. This assay is one of several recommended by EPA to identify, categorize and rank the pollutant potential of influent and effluent streams from industrial and energy-producing processes. This assay has been well validated with a wide range of positive and negative control chemicals and complex environmental samples.

All procedures and documents pertaining to the receipt, storage, preparation, testing and evaluation of the test material shall conform to Litton Bionetics, Inc. standard operating procedures and the Good Laboratory Practices Regulations of 1979. Deviations from standard procedure shall be fully documented and noted in the report.

All test and control results in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Molecular Toxicology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20895. Copies of raw data will be supplied to the sponsor upon request.

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5-256

I. ASSAY SUMMARY

A. Sponsor: Acurex Corporation

B. Material (Test Compound): Genetics Assay Number: 5883

1. Identification: A81-05-030-672 (EA-2 10+3)
2. Date Received: August 26, 1981
3. Physical Description: Fine, gray powder.

C. Type of Assay: EPA Level 1 Ames Salmonella/Microsome Plate Test

D. Assay Design Number: 401 (EPA Level 1)

E. Study Dates:

1. Initiation: October 26, 1981
2. Completion: November 9, 1981

F. Supervisory Personnel:

A. Study Director: D.R. Jagannath, Ph.D.

G. Evaluation:

The test material, A81-05-030-672 (EA-2 10+3), was tested for activity in the Ames Salmonella mutagenicity assay over a concentration range of 0.05 mg/plate to 5.0 mg/plate. The test was performed in duplicate under nonactivation and activation test conditions with strains TA-1535, TA-1537, TA-98, and TA-100.

The sample was not mutagenic under the test conditions employed and was ranked as having nondetectable (ND) mutagenic activity as defined by the IERL-EPA Level 1 criteria for the Ames bioassay[1].

Submitted by:

Study Director

11/24/81
D.R. Jagannath, Ph.D. Date
Section Chief,
Submammalian Genetics,
Department of Molecular
Toxicology

Reviewed by:

11/24/81
David J. Brusick, Ph.D. Date
Director,
Department of Molecular
Toxicology

II. OBJECTIVE

The objective of this study was to determine the genetic activity of A81-05-030-672 (EA-2 10+3) in the _Salmonella_/ microsome assay with and without the addition of mammalian metabolic activation preparations. The genetic activity of a sample is measured in these assays by its ability to revert the _Salmonella_ indicator strains from histidine dependence to histidine independence. The degree of genetic activity of a sample is reflected in the number of revertants that are observed on the histidine-free medium.

III. TEST MATERIAL

A. Description

The test material was received as a fine gray powder (1.5 gm) and was used without further preparation. The sample consisted of the 3 μm and 10 μm SASS train particulate catch.

B. Handling and Preparation

The test material was received at LBI on August 26, 1981. The sample was assigned LBI safety number 7167 and LBI assay number 5883. The sample was stored at +4°C in the dark.

A total of 476.58 mg of test material were used for two trials of the Ames *Salmonella* Assay. The test material was suspended at 100 mg/ml in dimethylsulfoxide (DMSO). The sample formed an opaque suspendion that settled upon standing. The suspension was incubated at 37°C on a shaker overnight to help leach material out of the particulates. Serial dilutions were made in DMSO such that 50 μl aliquots of each dilution give the desired concentration. The suspension was well mixed when aliquots were removed for dosing.

IV. MATERIALS

A. Indicator Microorganisms

The Salmonella typhimurium strains used in this assay were obtained from Dr. Bruce Ames, University of California at Berkeley.[4-8] The following four strains were used.

Strain Designation	Gene Affected	Additional Mutations			Mutation Type Detected
		Repair	LPS	R Factor	
TA-1535	his G	Δ uvr B	rfa	-	Base-pair substitution
TA-1537	his C	Δ uvr B	rfa	-	Frameshift
TA-98	his D	Δ uvr B	rfa	pKM101	Frameshift
TA-100	his G	Δ uvr B	rfa	pKM101	Base-pair substitution

All the above strains have, in addition to the mutation in the histidine operon, mutation (rfa-) that leads to defective lipopolysaccharide coat, a deletion that covers genes involved in the synthesis of vitamin biotin (bio-) and in the repair of ultraviolet (uv) - induced DNA damage (uvrB-). The rfa- mutation makes the strains more permeable to many large molecules. The uvrB- mutation decreases repair of some types of chemically or physically damaged DNA and thereby enhances the strain's sensitivity to some mutagenic agents. The resistant transfer factor plasmid (R factor) pKM101 in TA-98 and TA-100 is believed to cause an increase in error-prone DNA repair that leads to many more mutations for a given dose of most mutagens.[8] In addition, plasmid pKM101 confers resistance to the antibiotic ampicillin, which is a convenient marker to detect the presence of plasmid in the cells.

All indicator strains are kept at 4°C on minimal medium plates supplemented with a trace of biotin and an excess of histidine. In addition, the plates with plasmid-carrying strains contain ampicillin (25 µg/ml) to ensure stable maintenance of plasmid pKM101. New stock culture plates are made as often as necessary from the frozen master cultures or from single colony reisolates that were checked for their genotypic characteristics (his, rfa uvrB, bio) and for the presence of plasmid. For each experiment, an inoculum from the stock culture plates is grown overnight at 37°C in nutrient broth (Oxoid CM67) and used.

B. Media

The bacterial strains were cultured in Oxoid Media #2 (Nutrient Broth). The selective medium was Vogen Bonner Medium E with 2% glucose.[10] The

overlay agar consisted of 0.6% purified agar with 0.05 mM histidine, 0.05 mM biotin and 0.1M NaCl according to the methods of Ames _et al._[9]

C. Activation System

1. S9 Homogenate

A 9,000 x _g_ supernatant prepared from Sprague-Dawley adult male rat liver induced by Aroclor 1254 (Ames _et al._[9]) was purchased commercially and used in these assays.

2. S9 Mix

S9 mix used in these assays consisted of the following components:

Components	Concentration per Milliliter S9 Mix
NADP (sodium salt)	4 μmoles
D-glucose-6-phosphate	5 μmoles
$MgCl_2$	8 μmoles
KCl	33 μmoles
Sodium phosphate buffer pH 7.4	100 μmoles
Organ homogenate from rat liver (S9 fraction)	100 μliters

V. EXPERIMENTAL DESIGN

A. Dosage Selection

Test strategy and dose selection depend upon sample type and sample availability. The Level 1 manual[1] recommends solids to be initially tested at the maximum applicable dose (MAD) of 5 mg per plate and at lower concentrations of 2.5, 1, 0.5, 0.1 and 0.05 mg per plate. Liquids are tested initially at the MAD of 200 µl per plate, and at lower concentrations of 100, 50 and 10 µl per plate. Samples are retested over a narrower range of concentrations with strains showing positive results initially. Alternate dose are employed if sample size is limiting or at the direction of the sponsor.

Doses selected to test this sample covered the recommended dose range for solids. The highest dose was at the MAD level of 5 mg per plate and included five lower dose levels of 2.5, 1, 0.5, 0.1, and 0.05 mg per plate. A repeat trial with strain TA-98 without activation used the same dose levels.

B. Mutagenicity Testing

The procedure used was based on the paper published by Ames et. al.[9] and was performed as follows:

1. Nonactivation Assay

To a sterile 13 x 100 mm test tube placed in a 43°C water bath the following was added in order:

- 2.00 ml of 0.6% agar containing 0.05 mM histidine and 0.05 mM biotin.
- 0.05 ml of a suspension of the test chemical to give the appropriate dose.
- 0.1 ml to 0.2 ml of indicator organism(s).
- 0.50 ml of 0.2M phosphate buffer, pH 7.4.

This mixture was swirled gently and then poured onto minimal agar plates (see IV B, Media). After the top agar had set, the plates were incubated at 37°C for approximately 2 days. The number of his+ revertant colonies growing on the plates were counted with an automatic colony counter and recorded.

2. Activation Assay

The activation assay was run concurrently with the nonactivation assay. The only difference was the addition of 0.5 ml of S9 mix (see IV C, Activation System) to the tubes in place of 0.5 ml of phosphate buffer which was added in nonactivation assays. All other details were similar to the procedure for nonactivation assays.

A detailed flow diagram for the plate incorporation assay is provided in Figure 1.

C. Control Compounds

A negative control consisting of the solvent used for the test material was also assayed concurrently with the test material. For negative controls, step 'b' of Nonactivation Assays was replaced by 0.05 ml of the solvent. The negative controls were employed for each indicator strain and were performed in the absence and presence of S9 mix. The solvent used to prepare the stock solution of the test material is given in the Results section of this report. All dilutions of the test material were made using this solvent. The amount of solvent used was equal to the maximum volume used to give the appropriate test dose.

Specific positive control compounds known to revert each strain were also used and assayed concurrently with the test material. The concentrations and specificities of these compounds to specific strains are given in the following table:

Assay	Chemical	Solvent	Concentration per plate (µg)	Salmonella Strains
Nonactivation	Sodium azide	Water	10.0	TA-1535, TA-100
	2-Nitrofluorene (NF)	Dimethylsulfoxide	10.0	TA-98
	9-aminoacridine (9AA)	Ethanol	50.0	TA-1537
Activation	2-anthramine (ANTH)	Dimethylsulfoxide	2.5	For all strains

D. Recording and Presenting Data

The number of colonies on each plate were counted and recorded on printed forms. These raw data were analyzed in a computer program and reported on a printout. The results are presented as revertants per plate for each indicator strain employed in the assay. The positive and solvent controls are provided as reference points.

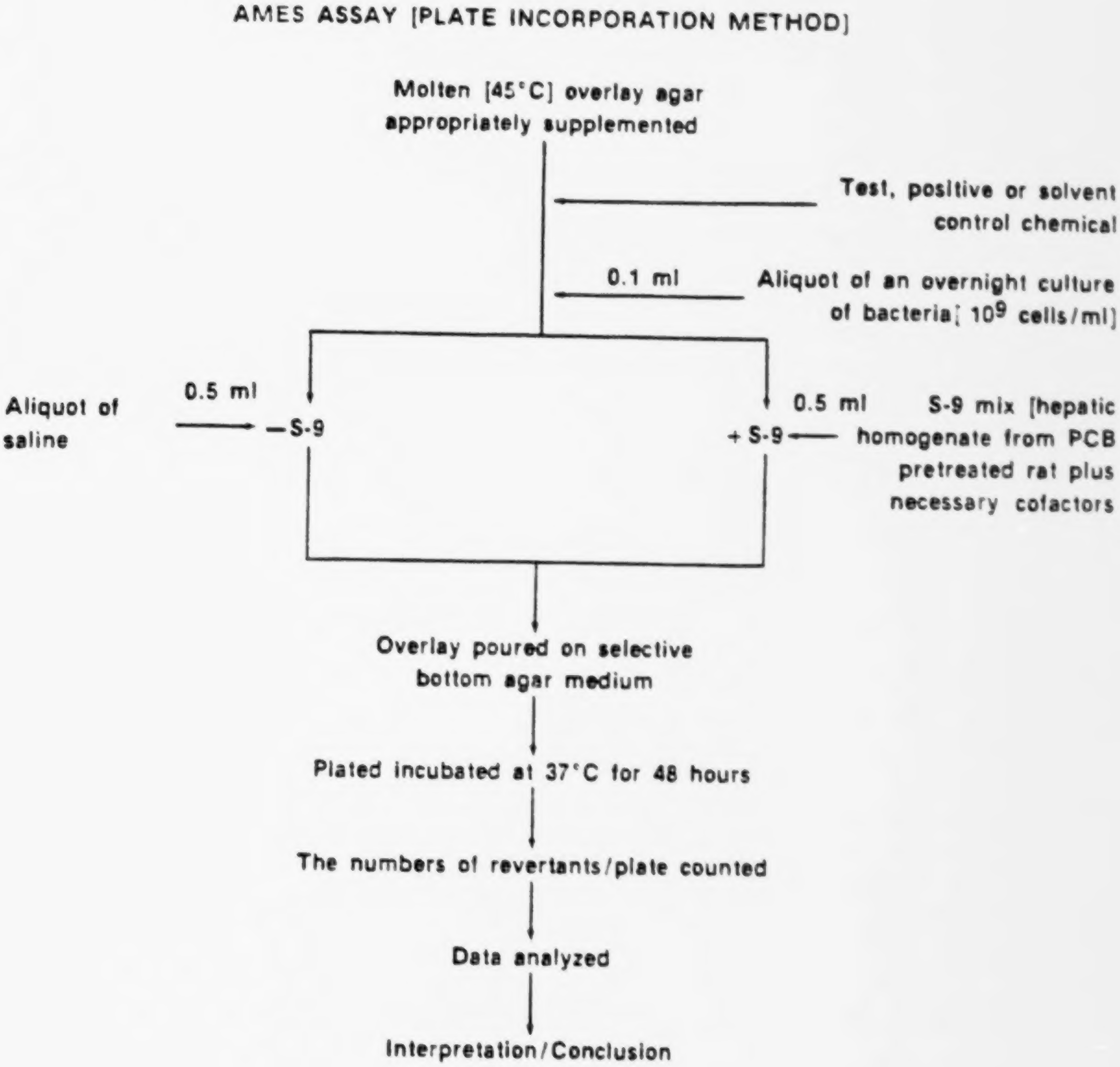
AMES ASSAY [PLATE INCORPORATION METHOD]
Molten [45°C] overlay agar appropriately supplemented
Test, positive or solvent control chemical
0.1 ml
Aliquot of an overnight culture of bacteria[10⁹ cells/ml]
Aliquot of saline
0.5 ml
—S-9
0.5 ml
+S-9
S-9 mix [hepatic homogenate from PCB pretreated rat plus necessary cofactors
Overlay poured on selective bottom agar medium
Plated incubated at 37°C for 48 hours
The numbers of revertants/plate counted
Data analyzed
Interpretation/Conclusion

Figure 1 AMES SALMONELLA/MICROSOME MUTAGENESIS ASSAY

VI. RESULTS

A. Interpretations

The test material, A81-05-030-672 (EA-2 10+3), was dissolved in DMSO at a stock concentration of 100 mg/ml and leached overnight on a shaker at 37°C. Additional dilutions were prepared in DMSO for testing. The maximum test level was 5.0 mg/plate. There was no evidence of toxicity at this level.

Reverse mutation was measured in strains TA-1535, TA-1537, TA-98 and TA-100. The test was conducted in duplicate both with and without rat liver S9 mix for metabolic activation. The trial with strain TA-98 without activation was repeated using the same test conditions, because in the first trial, one of the positive control plates was lost due to contamination.

There was no mutagenic activity associated with the test material treatment and the sample was considered nonmutagenic and non toxic. The sample was ranked as having nondetectable (ND) mutagenic activity using the IERL-EPA Level 1 evaluation criteria for the Ames Assay[1].

Solvent control and positive control values were within acceptable ranges. These results achieved assay acceptance criteria and provided confidence in the assumptions that the recorded data represented typical responses to the test material.

B. Tables

This report is based on the data provided in Tables 1 and 2.

RESULTS

TABLE 1

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: A81-05-030-672 FA-2 10+3
B. SOLVENT: DMSO
C. TEST INITIATION DATES: 10/26/81
D. TEST COMPLETION DATE: 10/29/81
E. S-9 LOT#: REF050
NOTE: CONCENTRATIONS ARE GIVEN IN MILLIGRAMS PER PLATE

TEST		SPECIES	TISSUE	REVERTANTS PER PLATE TA-1535 1	 2	 3	 TA-1537 1	 2	 3	 TA-98 1	 2	 3	 TA-100 1	 2	 3
NONACTIVATION															
SOLVENT CONTROL		---	---	16	19		7	4		24	26		116	118	
POSITIVE CONTROL**		---	---	1239	1052		733	650		860	C		1196	1080	
TEST COMPOUND															
0.050	MG	---	---	10	16		9	8		24	30		144	104	
0.100	MG	---	---	12	14		12	10		27	21		128	129	
0.500	MG	---	---	15	20		14	5		26	33		131	87	
1.000	MG	---	---	10	12		6	12		34	23		117	92	
2.500	MG	---	---	13	4		10	6		28	25		105	72	
5.000	MG	---	---	11	14		12	14		22	30		78	86	
ACTIVATION															
SOLVENT CONTROL		RAT	LIVER	15	11		6	7		41	37		92	101	
POSITIVE CONTROL***		RAT	LIVER	479	509		459	445		645	1991		2371	1861	
TEST COMPOUND															
0.050	MG	RAT	LIVER	16	12		11	11		34	34		120	100	
0.100	MG	RAT	LIVER	8	9		8	12		27	26		103	87	
0.500	MG	RAT	LIVER	14	13		13	9		29	41		100	98	
1.000	MG	RAT	LIVER	8	10		4	8		33	43		101	112	
2.500	MG	RAT	LIVER	12	14		7	4		41	29		95	98	
5.000	MG	RAT	LIVER	12	9		9	13		30	33		93	103	

**

TA-1535	SODIUM AZIDE	10 UG/PLATE
TA-1537	9-AMINOACRIDINE	50 UG/PLATE
TA-98	2-NITROFLUORENE	10 UG/PLATE
TA-100	SODIUM AZIDE	10 UG/PLATE
SOLVENT	50 UL/PLATE	

***

TA-1535	2-ANTHRAMINE	2.5 UG/PLATE
TA-1537	2-ANTHRAMINE	2.5 UG/PLATE
TA-98	2-ANTHRAMINE	2.5 UG/PLATE
TA-100	2-ANTHRAMINE	2.5 UG/PLATE

C INDICATES CONTAMINATION

```
  RESULTS                                                    TABLE 2
-------------------------------

A.    NAME OR CODE DESIGNATION OF THE TEST COMPOUND:  A81-05-536-672 EA-2 10+3
B.    SOLVENT:  DMSO
C.    TEST INITIATION DATES:  11/03/81
D.    TEST COMPLETION DATE: 11/09/81
E.    S-9 LOT#: REF050
NOTE:   CONCENTRATIONS ARE GIVEN IN MILLIGRAMS    PER  PLATE

                                     R E V E R T A N T S   P E R   P L A T E
                                   -----------------------------------------------------------------------------
TEST                SPECIES TISSUE   TA-98
----                ------- ------  --------------
                                      1    2    3
NONACTIVATION
-------------


SOLVENT CONTROL     ---     ---      22   23
POSITIVE CONTROL**  ---     ---    1128 1143

TEST COMPOUND
      0.050    MG   ---     ---      25   33
      0.100    MG   ---     ---      23   20
      0.500    MG   ---     ---      19   22
      1.000    MG   ---     ---      14   20
      2.500    MG   ---     ---      23   29
      5.000    MG   ---     ---      18   21
--------------------------------------------------------------------------------------------------------------------

**
   TA-98    2-NITROFLUORENE                           10 UG/PLATE
   SOLVENT  50 UL/PLATE
```

VII. ASSAY ACCEPTANCE AND EVALUATION CRITERIA

Statistical methods are not currently used, and evaluation is based on the criteria included in this protocol.

Plate test data consists of direct revertant colony counts obtained from a set of selective agar plates seeded with populations of mutant cells suspended in a semisolid overlay. Because the test material and the cells are incubated in the overlay for approximately 2 days and a few cell divisions occur during the incubation period, the test is semiquantitative in nature. Although these features of the assay reduce the quantitation of results, they provide certain advantages not contained in a quantitative suspension test:

- The small number of cell divisions permits potential mutagens to act on replication DNA, which is often more sensitive than nonreplicating DNA.
- The combined incubation of the test article and the cells in the overlay permits constant exposure of the indicator cells for approximately 2 days.

A. Surviving Populations

Plate test procedures do not permit exact quantitation of the number of cells surviving chemical treatment. At low concentrations of the test material, the surviving population on the treatment plates is essentially the same as that on the negative control plate. At high concentrations, the surviving population is usually reduced by some fraction. Our protocol will normally employ several doses ranging over two or three log concentrations, the highest of these doses being selected to show slight toxicity as determined by subjective criteria.

B. Dose-Response Phenomena

The demonstration of dose-related increased in mutant counts is an important criterion in establishing metagenicity. A factor that might modify dose-response results for a mutagen would be the selection of doses that are too low (usually mutagenicity and toxicity are related). If the highest dose is far lower than a toxic concentration, no increases may be observed over the dose range selected. Conversely, if the lowest dose employed is highly cytotoxic, the test material may kill any mutants that are induced, and the test material will not appear to be mutagenic.

C. Control Tests

Positive and negative control assays were conducted with each experiment and consisted of direct-acting mutagens for nonactivation assays and mutagens that require metabolic biotransformation in activation assays.

Negative controls consisted of the test material solvent in the overlay agar together with the other essential components. The negative control plate for each strain gave a reference point to which the test data was compared. The positive control assay was conducted to demonstrate that the test systems were functional with known mutagens.

The following normal range of revertants for solvent controls are generally considered acceptable.

TA-1535:	8-30
TA-1537:	4-30
TA-98:	20-75
TA-100:	80-250

D. Evaluation Criteria for Ames Assay

Because the procedures to be used to evaluate the mutagenicity of the test material are semiquantitative, the criteria to be used to determine positive effects are inherently subjective and are based primarily on a historical data base. Most data sets will be evaluated using the following criteria.

1. Strains TA-1535 and TA-1537

If the solvent control value is within the normal range, a test material that produces a positive dose response over three concentrations with the highest increase equal to three times the solvent control value will be considered to be mutagenic.

2. Strains TA-98 and TA-100

If the solvent control value is within the normal range, a test material that produces a positive dose response over three concentrations with the highest increase equal to twice the solvent control value for TA-98 and TA-100 will be considered to be mutagenic.

3. Pattern

Because TA-1535 and TA-100 are both derived from the same parental strain (G-46), to some extent there is a built-in redundancy in the microbial assay. In general, the two strains of a set respond to the same mutagen and such a pattern is sought. Generally, if a strain responds to a mutagen in nonactivation tests, it will do so in activation tests.

4. Reproducibility

If a test material produces a response in a single test that cannot be reproduced in additional runs, the initial positive test data lose significance.

The preceding criteria are not absolute, and other extenuating factors may enter into a final evaluation decision. However, these criteria will be applied to the majority of situations and are presented to aid those individuals not familar with this procedure. As the data base is increased, the criteria for evaluation can be more firmly established.

E. Relation Between Mutagenicity and Carcinogenicity

It must be emphasized that the Ames *Salmonella*/Microsome Plate Assay is not a definitive test for chemical carcinogens. It is recognized, however, that correlative and functional relations have been demonstrated between these two endpoints. The results of comparative tests on 300 chemicals by McCann *et al.*[4] show an extremely good correlation between results of microbial mutagenesis tests and *in vivo* rodent carcinogenesis assays.

All evaluations and interpretation of the data to be presented in the final report will be based only on the demonstration, or lack, of mutagenic activity.

F. Criteria for Ranking Samples in the Ames Assay

The goal of EPA Level 1 Ames testing is to rank source streams by relative degree of genetic toxicity (mutagenicity). Samples are first identified as mutagenic or nonmutagenic by the criteria in Section D above and then ranked using the mutagenicity categories presented in the table below. The lowest concentration giving a positive response in any strain, with or without metabolic activation, is identified as the minimum effective concentration (MEC) for that sample. The mutagenicity of the sample is evaluated as high (H), moderate (M), low (L), or nondetectable (ND) according to the evaluation criteria developed in the Level 1 manual[1] and summarized below. Samples with no detectable activity at the maximum applicable dose (MAD) are ranked nondetectable (ND).

Ames Assay Mutagenicity Ranking Criteria[1]

Mutagenic Activity	Solids (MEC in μg/plate)	Liquids[a] (MEC in μl/plate)
High (H)	<50	<2
Moderate (M)	50-500	2-20
Low (L)	500-5000	20-200
Not Detectable (ND)	>5000	>200

[a] Concentration of organic extracts is based upon organic content (μg organics per plate) and not volume (μl extract per plate) of sample tested.

VIII. REFERENCES

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GENETICS ASSAY NO.: 5883
LBI SAFETY NO.: 7167

CYTOTOXIC EVALUATION OF A81-05-030-672 (EA-2 10+3) IN THE RABBIT ALVEOLAR MACROPHAGE (RAM) CYTOTOXICITY ASSAY

FINAL REPORT

SUBMITTED TO:

ACUREX CORPORATION
485 CLYDE AVENUE
MOUNTAIN VIEW, CALIFORNIA 94042

SUBMITTED BY:

LITTON BIONETICS, INC.
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LBI PROJECT NO.: 22064

REPORT DATE: NOVEMBER 1981

Litton BIONETICS

PREFACE

This assay conforms to the standard EPA Level 1 procedure for the rabbit alveolar macrophage (RAM) cytotoxicity assay as described in "IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests" (1). The data were evaluated and formatted as recommended in "Level 1 Biological Testing Assessment and Data Formatting" (2).

The RAM cytotoxicity assay has been shown to be a sensitive method for detecting cytotoxic activity for a variety of chemicals representing various chemical classes (3). This assay is one of several recommended by EPA to identify, categorize and rank the pollutant potential of influent and effluent streams from industrial and energy-producing processes. This assay has been well validated with a wide range of positive and negative control chemicals and complex environmental samples.

All procedures and documents pertaining to the receipt, storage, preparation, testing and evaluation of the test material shall conform to Litton Bionetics, Inc. standard operating procedures and the Good Laboratory Practices Regulations of 1979. Deviations from standard procedure shall be fully documented and noted in the report.

All test and control results in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Molecular Toxicology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20895. Copies of raw data will be supplied to the sponsor upon request.

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I. ASSAY SUMMARY

A. SPONSOR: Acurex Corporation

B. MATERIAL (TEST COMPOUND): GENETICS ASSAY NUMBER: 5883

1. Identification: A81-05-030-672 (EA-2 10+3)
2. Date Received: August 26, 1981
3. Physical Description: Fine, gray powder

C. TYPE OF ASSAY: Rabbit Alveolar Macrophage (RAM) Cytotoxicity Assay

D. ASSAY DESIGN NUMBER: 443

E. STUDY DATES:

1. Initiation: October 22, 1981
2. Completion: October 23, 1981

F. SUPERVISORY PERSONNEL:

1. Study Director: Brian Myhr, Ph.D.
2. Laboratory Supervisor: Robert Young, M.S.

G. EVALUATION:

The test material, which was tested as supplied, caused a dose-related increase in toxicity for concentrations above approximately 200 µg/ml. The viability index and ATP content assay parameters were about equally sensitive and indicated an EC_{50} would be achieved at approximately the maximum applicable dose (MAD) of 1000 µg/ml. Since toxicity was clearly observed in the low toxicity region of 100-1000 µg/ml, as defined by the IERL-EPA evaluation criteria, and the projected EC_{50} values were essentially on the borderline between the low and nondetectable toxicity regions, the test material was best evaluated as having low/nondetectable (L/ND) toxicity to cultured RAM cells.

Submitted by:

Study Director

Brian Myhr 11/24/81

Brian Myhr, Ph.D. Date
Associate Director,
Department of Molecular
Toxicology

11/24/81

David J. Brusick, Ph.D. Date
Director,
Department of Molecular
Toxicology

II. OBJECTIVE

The objective of this study was to determine and rank the cytotoxicity of A81-05-030-672 (EA-2 10+3) to cultured rabbit alveolar macrophage (RAM) cells. The measure of cytotoxicity was the reduction in cell viability and adenosine triphosphate (ATP) content of the cultures after a 20 hour exposure to the test material. At the conclusion of the exposure period, the number of viable cells and total ATP content in the treated cultures were compared to the corresponding values in unexposed control cultures. The concentration of test material that reduced each experimental parameter by 50% was estimated graphically and referred to as the EC50 value. Standard EPA Level 1 toxicity evaluation criteria for the RAM cytotoxicity assay were used to rank the toxicity potential of the test material based upon the most sensitive parameter.

III. TEST MATERIAL

A. Description

The test material was received as a fine, gray powder (1.5 gm). No information on particle size distribution or sampling parameters was provided.

B. Handling and Preparation

The test material was received on August 26, 1981, and was assigned LBI assay number 5883 and LBI safety number 7167. The sample was stored at +4°C in the dark.

Approximately 33 mg of test material was used as supplied, without grinding, for the assay. The test material was suspended in serum-free EMEM culture medium at a concentration of 2000 μg/ml and incubated at 37°C on a roller drum for 8 hours. A fine, gray suspension was formed that settled upon standing. No pH changes were noted. The suspension was serially diluted with EMEM (serum-free) and applied to the cultures at a maximum concentration of 1000 μg/ml in the presence of 10% serum.

IV. MATERIALS

A. Indicator Cells

The assay employed short-term primary cultures of alveolar macrophage cells obtained by lung lavage of a male New Zealand white rabbit (2.4 kg). The rabbit was maintained on Purina Lab Rabbit Chow 5321 and water *ad libitum* and was examined for the absence of respiratory illnesses prior to use.

B. Media

The cells were maintained and treated in Eagle's Minimum Essential Medium (EMEM) with Earle's salts and supplemented with 10% fetal bovine serum (heat-inactivated), 100 units/ml penicillin, 100 µg/ml streptomycin, 17.6 µg/ml kanamycin, and 0.4 µg/ml amphotericin B.

C. Negative Controls

The negative control consisted of three untreated cultures carried through the same experimental time period as the treated cells. The average viability and ATP content of the negative control provided the reference points for determining the effects of different concentrations of the test material on the assay parameters.

V. EXPERIMENTAL DESIGN

A. Procurement of Cells

A rabbit was sacrificed by injection of Nembutal® (60 mg/ml) into the marginal ear vein, and sterile operating techniques were used to perform a tracheostomy. Prewarmed normal saline (30 ml) was then introduced into the lungs via a catheter and allowed to stand for 15 minutes. This lavage fluid was removed and placed into a 50-ml sterile centrifuge tube on ice. Nine additional lavages were similarly performed and collected, except the saline was removed shortly after its introduction into the lungs. Any lavage fluid containing blood or mucous was discarded. The lavages were centrifuged at 365 x g for 15 minutes and the cells resuspended in cold 0.85% saline. After two washes in saline by centrifugation, the cell pellets were resuspended in cold EMEM containing 20% serum and then combined. A cell count was obtained by hemocytometer and the suspension diluted to between 5.13×10^5 and 10^6 cells/ml. Viability was determined by trypan blue staining and the cells were not used if less than 95% viable. Also, a differential cell count from Wright-stained smears was performed to verify that the macrophage content was above 90%.

B. Sample Forms

The usual sample form for application to the cells is a suspension of particulate material. Solid samples are ground to fine particles and a weighed portion is suspended in a known volume of EMEM (0% FBS) for about eight hours to help leach any water-soluble material. Finely-divided test material may be suspended directly in culture medium without further grinding. Aqueous liquids, suspensions, or slurries containing less than 0.5% organic solvent are added by volume to culture medium.

Samples supplied as solutions in organic solvents are usually solvent-exchanged into DMSO before testing. Original sample volumes may first be reduced a maximum of 10-fold in a Kuderna-Danish concentrator, and the concentrative factor is used to convert assayed volumes into equivalent original sample volumes in the absence of information about solute concentration. An aliquot of the reduced volume is exchanged into DMSO by repeated, partial evaporation under a stream of nitrogen in a warm water bath (50°C); the evaporated volumes are replaced with equal volumes of DMSO.

Samples adsorbed on XAD-2 resin are extracted with methylene chloride or acetone in a Soxhlet apparatus for 24 hours. The extract is then concentrated and solvent-exchanged into DMSO. Alternatively, acetone extracts can be assayed directly at concentrations up to 2% by volume in the culture medium.

Samples impregnated on fiber glass or teflon filters are repeatedly sonicated in cyclohexane to remove particulates. The resulting cyclohexane particulate suspension is then evaporated to dryness and the particulates resuspended in EMEM culture medium at the desired concentration.

Sponsor-specified handling of sample materials will be followed if the above procedures are not applicable or a specific procedure is desired.

C. Dose Selection

Unless the approximate toxicity is already known or the sample size is limiting, the following usual dose ranges are tested for different sample forms. Dry, particulate material is tested at six dose levels from 1000 µg/ml to 3 µg/ml. Aqueous samples, suspensions, or slurries are tested from 600 µl to 3 µl/ml in six dose steps. Samples that are solvent-exchanged into DMSO are tested from 20 µl/ml (2% DMSO in growth medium) to 0.2 µl/ml, also in six dose steps. A second dose study is performed with an adjusted dose range if the EC50 was not located properly in the initial test. However, EC50 values greater than 1000 µl/ml for particulate material, 600 µl/ml for aqueous samples, or 20 µl/ml for organic solutions will not be determined.

This test material, A81-05-030-672 (EA-2 10+3), was tested at 6 dose levels, starting at the maximum applicable dose (MAD) of 1000 µg/ml and including 600, 300, 100, 60 and 30 µg/ml.

D. Treatment

A series of 25 cm^2 culture flasks were prepared, each containing 2.0 ml of serum-free medium at 37°C and the test material at twice the desired final concentration. Three flasks were prepared for each test concentration. Aliquots of cell suspension (2 ml) were then added; each flask, therefore, contained from 1.03 to 2 x 10^6 viable cells in a 4-ml volume of media containing 10% serum. The flasks were placed on a rocker platform in a 37°C incubator with a humidified atmosphere containing 5% CO_2. After sitting for about 30 minutes, the flasks were slowly rocked for the remainder of a 20-hour exposure period.

If the test substance causes a color change in the growth medium, the pH is determined in additional treated flasks. After the exposure period, the pH of the medium in the experimental flasks is again recorded.

E. Cell Viability Assay

At the end of the treatment period, the medium containing unattached cells was decanted into a centrifuge tube on ice. The attached cells were rinsed with 1 ml of 0.1% trypsin/0.01% versene and then incubated with 2 ml of the trypsin/versene solution for about 5 minutes at 37°C. The trypsinates and decanted media were combined for each culture to yield a 7-ml cell suspension for subsequent analysis.

A 1.0 ml aliquot of the cell suspension was removed for cell count and viability determination. The aliquot was combined with 1.0 ml of 0.4% trypan blue and counted by hemocytometer about 5 to 15 minutes later. The total number of cells counted per culture was the sum of the numbers found in five squares for each chamber of the hemocytometer (1 µl total volume). The numbers of live (colorless) and dead (blue) cells were recorded.

F. ATP Assay

ATP was immediately analyzed by extraction of a 0.1-ml sample of cell suspension with 0.9 ml of 90% DMSO. After 2 minutes at room temperature 5.0 ml cold MOPS buffer (0.01 M morpholinopropane sulfonic acid) at pH 7.4 was added and the extract mixed well and placed on ice. Aliquots of 10 µl were injected into a cuvette containing a luciferin-luciferase reaction mixture in a DuPont Model 760 Luminescence Biometer. The Biometer was calibrated daily with standard ATP solutions to provide a direct read-out of the ATP content. Each test sample was assayed at least twice to obtain repeatable readings.

VI. ASSAY ACCEPTANCE CRITERIA

The assay will be considered acceptable for evaluation of the test results if the following criteria are met:

1. The macrophage population is 90% or greater of the total nucleated cells collected by lung lavage.

2. The percent viability of the macrophages used to initiate the assay is 95% or greater.

3. The survival of viable macrophages in the negative control cultures over the 20 hour treatment priod is 70% or greater.

4. A sufficient number of data points (for five test concentrations or less) are available to clearly locate the EC50 of the most sensitive test parameter within a toxicity region as defined under Assay Evaluation Criteria.

5. The data points critical to the location of the EC50 for the most sensitive parameter are the averages of at least two treated cultures.

6. If all the test parameters yield EC50 values greater than 1000 µg/ml, 600 µl/ml for aqueous solutions, or 20 µl/ml for organic solutions, the plotted curves for ATP content and viability index parameters do not exceed 120% of the negative control.

VII. RESULTS

A. Interpretation

The test material was ingested by the macrophages and caused a general decline in their viability when the applied concentration was increased above approximately 200 µg/ml. Absolute values for the assay parameters are given in Table 1, as well as the parameters relative to the negative control average value, and the relative values are plotted in Figures 1 and 2.

The viability index (which measures cell survival) and the culture ATP content usually tend to parallel each other, and an inspection of the results in Figures 1 and 2 show this to be the case for the current assay. Both parameters were about equally sensitive and showed declines in ATP and the numbers of viable cells in the 100-1000 µg/ml concentration range. Both parameters also indicated the EC_{50} values would be achieved for concentrations at or just above the MAD level of 1000 µg/ml. Therefore, strict application of the IERL-EPA evaluation criteria would result in a nondetectable toxicity classification. However, toxicity was clearly evident in the low toxicity region (100-1000 µg/ml), and repeat assays could be expected to result in variations in the EC_{50} positions such that borderline responses could fall within either the low or nondetectable categories. The percent viability and ATP/10^6 cells parameters were essentially nonresponsive and did not contribute to an evaluation of the test material. On the basis of the responsive parameters, the test material was evaluated as having low/nondetectable (L/ND) borderline toxicity to the RAM cells.

The macrophages collected for this assay had normal morphology and appeared to be in a healthy state. The initial viability was excellent (99.4%) and the survival of viable cells in the negative control was 98.9%. The average cellular ATP content of the negative controls 29.0 x 10^8 fg ATP per 10^6 total cells, which wash within the historical range for acceptable cultures. These results achieved the assay acceptance criteria and provided confidence in the assumption that the collected data represented typical responses to the test material.

B. Tables and Figures

This report is based on the data provided in Table 1 and Figures 1 and 2.

TABLE 1
RABBIT ALVEOLAR MACROPHAGE (RAM) CYTOTOXICITY ASSAY DATA

LBI Assay No.: 5883

Test Material Identity: A81-05-030-672 (EA-2 10+3)

Test Date: October 22, 1981

Vehicle: EMEM

Initial Cell Viability: 99.4%

Viable Macrophage Seeded/Flask: 1.03×10^6

Macrophage Population Percentage: >90%

Survival of Negative Control Macrophage Over Treatment Time: 98.9%

Sample	Concentration[a] µg/ml	Average Values per Culture Flask			ATP Per 10^6 Cells 10^8 fg	Viability %	Expressed as Percent of Negative Control			
		Viable Cells 10^6 Units	Total Cells 10^6 Units	ATP 10^8 fg[b]			Viability	Viability Index	ATP	ATP Per 10^6 Cells
NC[c]	---	0.89	0.90	26.1	29.0	98.9	100.0	100.0	100.0	100.0
TEST	30	0.90	0.91	26.1	28.7	98.9	100.0	101.1	100.0	99.0
TEST	60	0.97	0.98	25.7	26.2	99.0	100.1	109.0	98.5	90.3
TEST	100	0.86	0.90	24.5	27.2	95.6	96.7	96.6	93.9	93.8
TEST	300	0.63	0.67	22.5	33.6	94.0	95.0	70.8	86.2	115.9
TEST	600	0.49	0.54	17.4	32.2	90.7	91.7	55.1	66.7	111.0
TEST	1000	0.54	0.61	14.4	23.6	88.5	89.5	60.7	55.2	81.4

	Viability	Viability Index	ATP	ATP Per 10^6 Cells
[d]EC50 VALUES: µg/ml:	>1000	1000	>1000	>1000

Toxicity Classification: Low/Nondetectable Borderline

[a] pH change in culture medium: None observed

[b] fg = Femtogram (10^{-15} gram).

[c] NC = Negative Control, EMEM culture medium.

[d] Determined from data plots in Figures 1 and 2.

FIGURE 1

EC50 DETERMINATION FOR

PERCENT VIABILITY (O) AND VIABILITY INDEX (●)

A81-05-030-672

(EA-2 10+3)

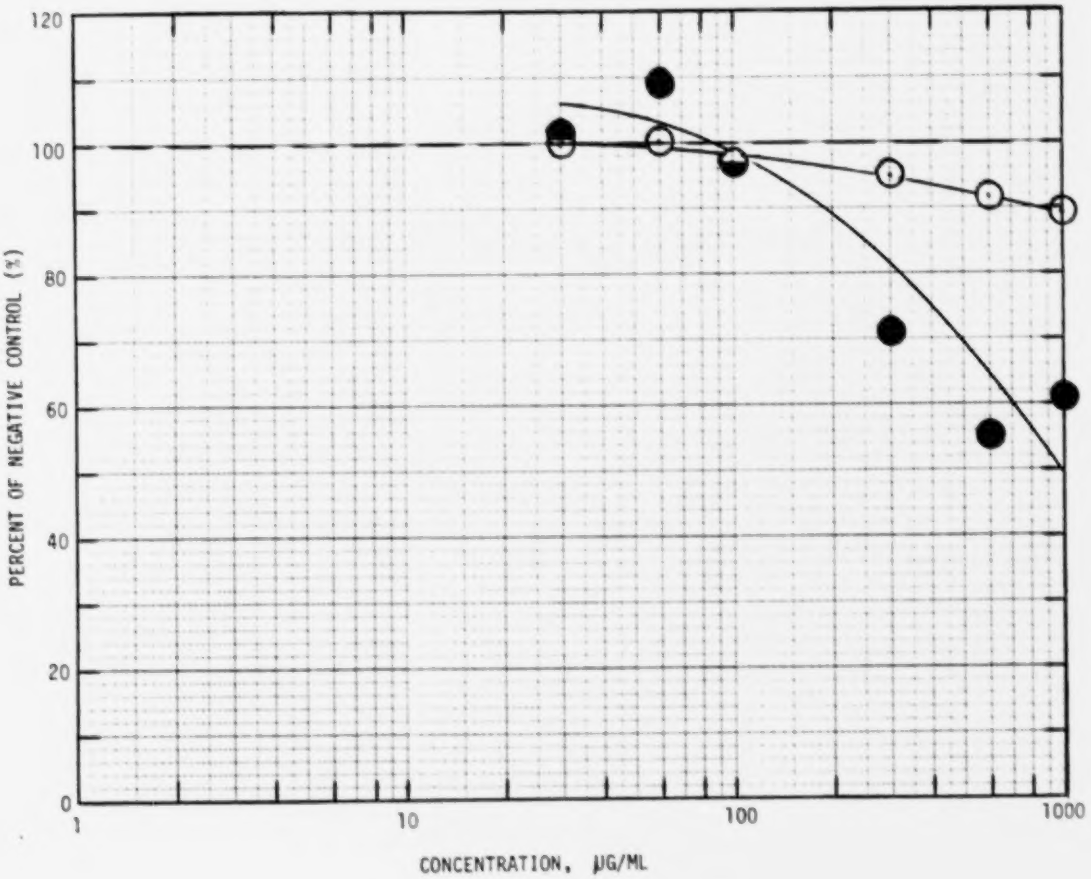


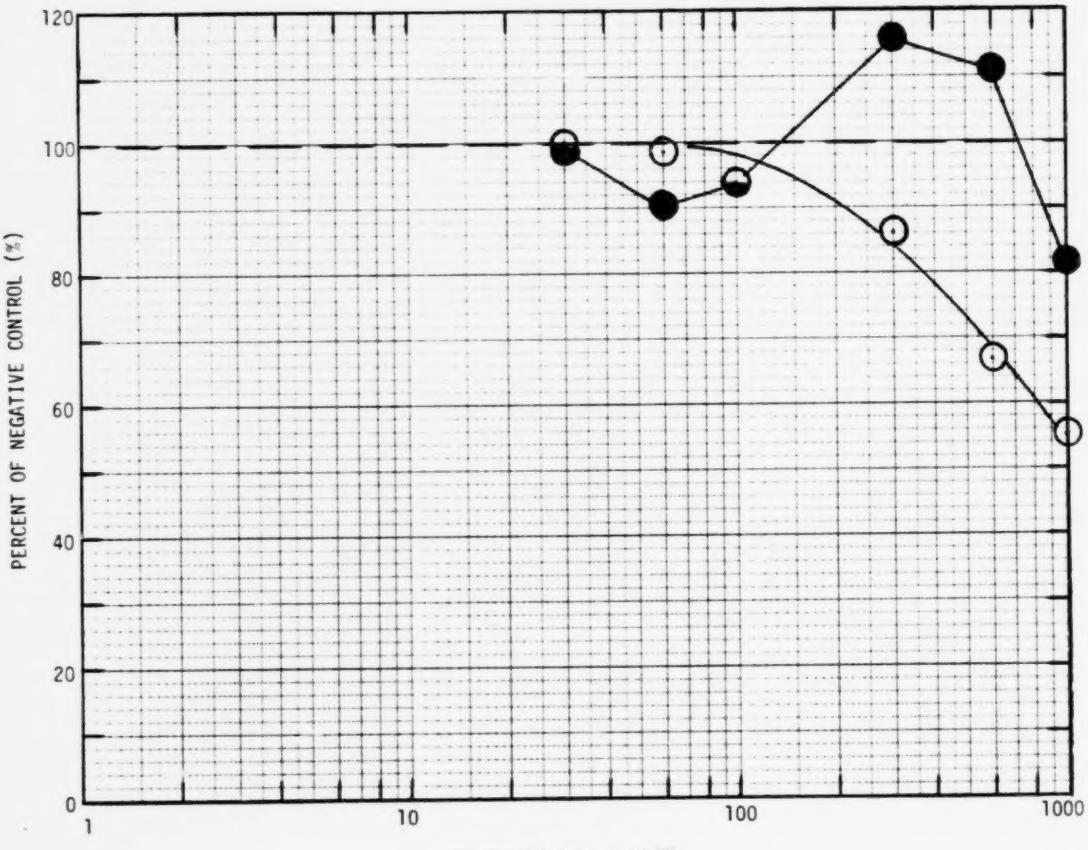
FIGURE 2

EC50 DETERMINATION FOR

ATP/FLASK (O) AND ATP/10^6 CELLS (●)

A81-05-030-672

(EA-2 10+3)



VIII. ASSAY EVALUATION CRITERIA

The EC50 value represents the concentration of test material that reduces the most sensitive parameter of the RAM assay to 50% of the vehicle or negative control value. EC50 values are determined graphically by fitting a curve by eye through relative toxicity data plotted as a function of the logarithm of the applied concentration. Each data point normally represents the average of three culture dishes. Statistical analysis is unnecessary in most cases for evaluation.

The toxicity of the test material is evaluated as high, moderate, low, or nondetectable according to the range of EC50 values defined in the following table.

Toxicity[a]	Solids (EC_{50} in μg/ml)	Aqueous Liquids (EC_{50} in μl/ml)	Nonaqueous Liquids[b] (EC_{50} in μl/ml)
High	<10	<6	<0.2
Moderate	10 to 100	6 to 60	0.2-2
Low	100 to 1000	60 to 600	2-20
Not Detectable	>1000	>600	>20

[a] Evaluation criteria formulated by Litton Bionetics, Inc. for IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests[1].

[b] Criteria for nonaqueous liquids are tentative and under evaluation. If the organic or solid content is known, the solid evaluation criteria are applied.

Another evaluation scheme is proposed for extracts obtained from SASS train gas volumes. The proportion of the total gas volume corresponding to the volume of extract used in the bioassay is calculated and expressed as L/ml of culture medium (or DSCF/ml of culture medium). A criterion of 1000 L/ml is set as the limit for nondetectable toxicity. This gas volume corresponds to the average volume breathed by humans over a 2-hour period. The subsequent toxicity ranges are defined by 10-fold dilution steps to conform to standard procedure. The toxicity ranges are defined in the following table for liter and dry standard cubic feet units:

Toxicity	EC_{50} In Liters/ml (L/ml)	EC_{50} In Dry Standard Cubic Feet/ml (DSCF/ml)
High	<10	<0.35 DSCF
Moderate	10-100	0.35-3.5
Low	100-1000	3.5-35
Nondetectable	>1000	>35

IX. REFERENCES

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2. Brusick, D.J.: Level 1 Bioassay Assessment and Data Formatting. EPA-600/7-80-079, Litton Bionetics, Inc., Kensington, MD, April 1980, 100 pp.

3. Brusick, D.J. and Young, R.R.: Level 1 Bioassay Sensitivity. EPA-600/7-81-135, Litton Bionetics, Inc., Kensington, MD, August 1981, pp. 52.

GENETICS ASSAY NO.: 5884
LBI SAFETY NO.: 7168

MUTAGENICITY EVALUATION OF A81-05-030-674 (EA-2 1+FILTER) IN THE EPA LEVEL 1 AMES SALMONELLA/MICROSOME PLATE TEST

FINAL REPORT

SUBMITTED TO:

ACUREX CORPORATION
485 CLYDE AVENUE
MOUNTAIN VIEW, CALIFORNIA 94042

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20895

LBI PROJECT NO.: 22064

REPORT DATE: NOVEMBER 1981

Litton BIONETICS

PREFACE

This assay conforms to the standard EPA Level 1 procedure for the Ames Salmonella/microsome mutagenesis assay as described in "IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests"[1]. The data were evaluated and formatted as recommended in "Level 1 Biological Testing Assessment and Data Formatting"[2].

The Ames Salmonella/microsome mutagenesis assay has been shown to be a sensitive method for detecting mutagenic activity for a variety of chemicals representing various chemical classes[3]. This assay is one of several recommended by EPA to identify, categorize and rank the pollutant potential of influent and effluent streams from industrial and energy-producing processes. This assay has been well validated with a wide range of positive and negative control chemicals and complex environmental samples.

All procedures and documents pertaining to the receipt, storage, preparation, testing and evaluation of the test material shall conform to Litton Bionetics, Inc. standard operating procedures and the Good Laboratory Practices Regulations of 1979. Deviations from standard procedure shall be fully documented and noted in the report.

All test and control results in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Molecular Toxicology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20895. Copies of raw data will be supplied to the sponsor upon request.

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I. ASSAY SUMMARY

A. Sponsor: Acurex Corporation

B. Material (Test Compound): Genetics Assay Number: 5884

1. Identification: A81-05-030-674 (EA-2 1+Filter)
2. Date Received: August 26, 1981
3. Physical Description: Fine, gray/black powder and fiberglass filter with embedded particles.

C. Type of Assay: EPA Level 1 Ames Salmonella/Microsome Plate Test

D. Assay Design Number: 401 (EPA Level 1)

E. Study Dates:

1. Initiation: October 1, 1981
2. Completion: October 29, 1981

F. Supervisory Personnel:

A. Study Director: D.R. Jagannath, Ph.D.

G. Evaluation:

The test material, A81-05-030-674 (EA-2 1+filter), was tested for activity in the Ames *Salmonella* mutagenicity assay over a concentration range of 0.05 mg/plate to 5.0 mg/plate. The test was performed in duplicate under nonactivation and activation test conditions with strains TA-1535, TA-1537, TA-98, and TA-100.

The sample was not mutagenic under the test conditions employed and was ranked as having nondetectable (ND) mutagenic activity as defined by the IERL-EPA Level 1 criteria for the Ames bioassay[1].

Submitted by:

Study Director

DR Jagannath 11/24/81

D.R. Jagannath, Ph.D. Date
Section Chief,
Submammalian Genetics,
Department of Molecular
Toxicology

Reviewed by:

11/[illegible]

David J. Brusick, Ph.D. Date
Director,
Department of Molecular
Toxicology

II. OBJECTIVE

The objective of this study was to determine the genetic activity of A81-05-030-674 (EA-2 1+Filter) in the *Salmonella*/ microsome assay with and without the addition of mammalian metabolic activation preparations. The genetic activity of a sample is measured in these assays by its ability to revert the *Salmonella* indicator strains from histidine dependence to histidine independence. The degree of genetic activity of a sample is reflected in the number of revertants that are observed on the histidine-free medium.

III. TEST MATERIAL

A. Description

The test material, as received, was comprised to two separate components. The first component, a fine, gray/black powder, was the 1 µm SASS train particulate catch. The second component was a fiberglass filter with embedded particulate material. This gray/black particulate material represented particulates less than 1 µm collected in the SASS train sample. Both components were supplied together in a Nalgene® screw-top bottle.

B. Handling and Preparation

The test material was received at LBI on August 26, 1981. The sample was assigned LBI safety number 7168 and LBI assay number 5884. The sample was stored at +4°C in the dark.

The filter portion of the sample required removal of the embedded particulates before testing could begin. The uncut filter was sonicated in cyclohexane as recommended by current IERL-EPA pretest sample preparation procedures[1]. The decanted particulate suspension from three successive sonication treatments were combined and evaporated to dryness. The particulate material was weighed and combined with the 1 µm particulate catch portion of the sample. A total of 264.42 mg of the combined test material available for testing was comprised of 70.28 mg (26.6%) of <1 µm particulates removed from the filter and 194.14 mg (73.4%) of 1 µm particulates.

Approximately 220 mg of the test material were used for the trial in the Ames Salmonella Assay. The test material was suspended at 100 mg/ml in dimethylsulfoxide (DMSO) and incubated overnight at 37°C on a rotary shaker. This stock suspension was used to make dilutions in DMSO to be used for dosing in the EPA Level 1 Ames Salmonella Assay.

IV. MATERIALS

A. Indicator Microorganisms

The *Salmonella typhimurium* strains used in this assay were obtained from Dr. Bruce Ames, University of California at Berkeley.[4-8] The following four strains were used.

Strain Designation	Gene Affected	Additional Mutations			Mutation Type Detected
		Repair	LPS	R Factor	
TA-1535	*his* G	Δ *uvr* B	*rfa*	-	Base-pair substitution
TA-1537	*his* C	Δ *uvr* B	*rfa*	-	Frameshift
TA-98	*his* D	Δ *uvr* B	*rfa*	pKM101	Frameshift
TA-100	*his* G	Δ *uvr* B	*rfa*	pKM101	Base-pair substitution

All the above strains have, in addition to the mutation in the histidine operon, mutation (*rfa*-) that leads to defective lipopolysaccharide coat, a deletion that covers genes involved in the synthesis of vitamin biotin (*bio*-) and in the repair of ultraviolet (uv) - induced DNA damage (*uvrB*-). The *rfa*- mutation makes the strains more permeable to many large molecules. The *uvrB*- mutation decreases repair of some types of chemically or physically damaged DNA and thereby enhances the strain's sensitivity to some mutagenic agents. The resistant transfer factor plasmid (R factor) pKM101 in TA-98 and TA-100 is believed to cause an increase in error-prone DNA repair that leads to many more mutations for a given dose of most mutagens.[8] In addition, plasmid pKM101 confers resistance to the antibiotic ampicillin, which is a convenient marker to detect the presence of plasmid in the cells.

All indicator strains are kept at 4°C on minimal medium plates supplemented with a trace of biotin and an excess of histidine. In addition, the plates with plasmid-carrying strains contain ampicillin (25 µg/ml) to ensure stable maintenance of plasmid pKM101. New stock culture plates are made as often as necessary from the frozen master cultures or from single colony reisolates that were checked for their genotypic characteristics (*his*, *rfa* *uvrB*, *bio*) and for the presence of plasmid. For each experiment, an inoculum from the stock culture plates is grown overnight at 37°C in nutrient broth (Oxoid CM67) and used.

B. Media

The bacterial strains were cultured in Oxoid Media #2 (Nutrient Broth). The selective medium was Vogen Bonner Medium E with 2% glucose.[10] The

overlay agar consisted of 0.6% purified agar with 0.05 mM histidine, 0.05 mM biotin and 0.1M NaCl according to the methods of Ames et al.[9]

C. Activation System

1. S9 Homogenate

A 9,000 x g supernatant prepared from Sprague-Dawley adult male rat liver induced by Aroclor 1254 (Ames et al.[9]) was purchased commercially and used in these assays.

2. S9 Mix

S9 mix used in these assays consisted of the following components:

Components	Concentration per Milliliter S9 Mix
NADP (sodium salt)	4 μmoles
D-glucose-6-phosphate	5 μmoles
$MgCl_2$	8 μmoles
KCl	33 μmoles
Sodium phosphate buffer pH 7.4	100 μmoles
Organ homogenate from rat liver (S9 fraction)	100 μliters

V. EXPERIMENTAL DESIGN

A. Dosage Selection

Test strategy and dose selection depend upon sample type and sample availability. The Level 1 manual[1] recommends solids to be initially tested at the maximum applicable dose (MAD) of 5 mg per plate and at lower concentrations of 2.5, 1, 0.5, 0.1 and 0.05 mg per plate. Liquids are tested initially at the MAD of 200 µl per plate, and at lower concentrations of 100, 50 and 10 µl per plate. Samples are retested over a narrower range of concentrations with strains showing positive results initially. Alternate dose are employed if sample size is limiting or at the direction of the sponsor.

Doses selected to test this sample covered the recommended dose range for solids. The highest dose was at the MAD level of 5 mg per plate and included five lower dose levels of 2.5, 1, 0.5, 0.1 and 0.05 mg per plate.

B. Mutagenicity Testing

The procedure used was based on the paper published by Ames *et. al.*[9] and was performed as follows:

1. Nonactivation Assay

To a sterile 13 x 100 mm test tube placed in a 43°C water bath the following was added in order:

- 2.00 ml of 0.6% agar containing 0.05 mM histidine and 0.05 mM biotin.
- 0.05 ml of a suspension of the test chemical to give the appropriate dose.
- 0.1 ml to 0.2 ml of indicator organism(s).
- 0.50 ml of 0.2M phosphate buffer, pH 7.4.

This mixture was swirled gently and then poured onto minimal agar plates (see IV B, Media). After the top agar had set, the plates were incubated at 37°C for approximately 2 days. The number of *his*+ revertant colonies growing on the plates were counted with an automatic colony counter and recorded.

2. Activation Assay

The activation assay was run concurrently with the nonactivation assay. The only difference was the addition of 0.5 ml of S9 mix (see IV C, Activation System) to the tubes in place of 0.5 ml of phosphate buffer which was added in nonactivation assays. All other details were similar to the procedure for nonactivation assays.

A detailed flow diagram for the plate incorporation assay is provided in Figure 1.

C. Control Compounds

A negative control consisting of the solvent used for the test material was also assayed concurrently with the test material. For negative controls, step 'b' of Nonactivation Assays was replaced by 0.05 ml of the solvent. The negative controls were employed for each indicator strain and were performed in the absence and presence of S9 mix. The solvent used to prepare the stock solution of the test material is given in the Results section of this report. All dilutions of the test material were made using this solvent. The amount of solvent used was equal to the maximum volume used to give the appropriate test dose.

Specific positive control compounds known to revert each strain were also used and assayed concurrently with the test material. The concentrations and specificities of these compounds to specific strains are given in the following table:

Assay	Chemical	Solvent	Concentration per plate (μg)	Salmonella Strains
Nonactivation	Sodium azide	Water	10.0	TA-1535, TA-100
	2-Nitrofluorene (NF)	Dimethyl-sulfoxide	10.0	TA-98
	9-aminoacridine (9AA)	Ethanol	50.0	TA-1537
Activation	2-anthramine (ANTH)	Dimethyl-sulfoxide	2.5	For all strains

D. Recording and Presenting Data

The number of colonies on each plate were counted and recorded on printed forms. These raw data were analyzed in a computer program and reported on a printout. The results are presented as revertants per plate for each indicator strain employed in the assay. The positive and solvent controls are provided as reference points.

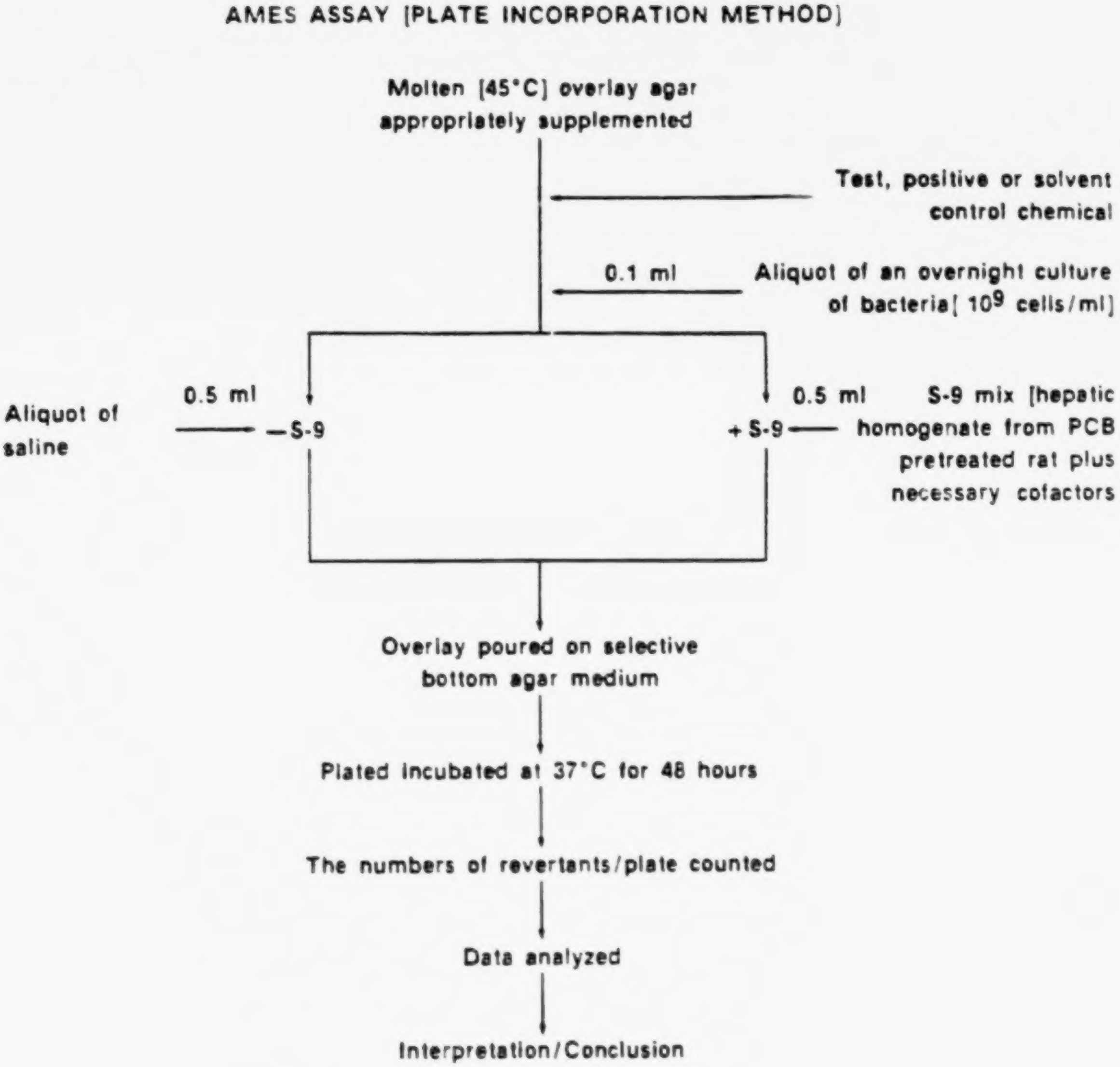
AMES ASSAY [PLATE INCORPORATION METHOD]
Molten [45°C] overlay agar appropriately supplemented
Test, positive or solvent control chemical
0.1 ml
Aliquot of an overnight culture of bacteria[10^9 cells/ml]
Aliquot of saline
0.5 ml
—S-9
0.5 ml
+S-9
S-9 mix [hepatic homogenate from PCB pretreated rat plus necessary cofactors
Overlay poured on selective bottom agar medium
Plated incubated at 37°C for 48 hours
The numbers of revertants/plate counted
Data analyzed
Interpretation/Conclusion

Figure 1 AMES SALMONELLA/MICROSOME MUTAGENESIS ASSAY

VI. RESULTS

A. Interpretations

The test material, A81-05-030-674 (EA-2 1+Filter), was dissolved in DMSO at a stock concentration of 100 mg/ml and leached overnight on a shaker at 37°C. Additional dilutions were prepared in DMSO for testing. The maximum test level was 5.0 mg/plate. There was no evidence of toxicity at this level.

Reverse mutation was measured in strains TA-1535, TA-1537, TA-98 and TA-100. The test was conducted in duplicate both with and without rat liver S9 mix for metabolic activation.

There was no mutagenic activity associated with the test material treatment and the sample was considered nonmutagenic and non toxic. The sample was ranked as having nondetectable (ND) mutagenic activity using the IERL-EPA Level 1 evaluation criteria for the Ames Assay[1].

Solvent control and positive control values were within acceptable ranges. These results achieved assay acceptance criteria and provided confidence in the assumptions that the recorded data represented typical responses to the test material.

B. Tables

This report is based on the data provided in Table 1.

```
RESULTS                                              TABLE 1
--------------------------------

A.    NAME OR CODE DESIGNATION OF THE TEST COMPOUND:  A-81-05-030-674(EA-2 1+FILTER)
B.    SOLVENT:  DMSO
C.    TEST INITIATION DATES:  10/26/81
D.    TEST COMPLETION DATE: 10/29/81
E.    S-9 LOT#: REF050
NOTE:   CONCENTRATIONS ARE GIVEN IN MILLIGRAMS    PER  PLATE

                                     R E V E R T A N T S   P E R   P L A T E
                                   ---------------------------------------------------------------------------------
TEST                  SPECIES TISSUE  TA-1535        TA-1537         TA-98          TA-100
----                  ------- ------ -------------- -------------- -------------- --------------
                                      1    2    3     1    2    3     1    2    3     1    2    3
NONACTIVATION
-------------


SOLVENT CONTROL         ---     ---   16   19          7    4         24   26         116  118
POSITIVE CONTROL**      ---     ---  1239 1052        733  653        860    C        1196 1080

TEST COMPOUND
      0.050000 MG       ---     ---   11   10         14    7         34   24         106  105
      0.100000 MG       ---     ---   10    8          9   11         20   21         125  113
      0.500000 MG       ---     ---   22   15          9   10         32   42         116  138
      1.000000 MG       ---     ---   17   15         10    6         35   29         131  119
      2.500000 MG       ---     ---   21   23         13   16         48   46         136  135
      5.000000 MG       ---     ---   23   23         18   21          0   34         142  132
ACTIVATION
----------


SOLVENT CONTROL       RAT     LIVER   15   11          6    7         41   37          92  101
POSITIVE CONTROL***   RAT     LIVER  479  509        459  445        645 1991        2371 1661

TEST COMPOUND
      0.050000 MG     RAT     LIVER   10    8         17    5         36   20          89  108
      0.100000 MG     RAT     LIVER   11   17          9   11         39   40         103  100
      0.500000 MG     RAT     LIVER   11    8          8   14         39   35         124  114
      1.000000 MG     RAT     LIVER   14   13         13   14         44   30         119  113
      2.500000 MG     RAT     LIVER   11   17         16   10         46   34         148  116
      5.000000 MG     RAT     LIVER   16   17         15   21         40   45         124  114
--------------------------------------------------------------------------------------------------------
**                                                        ***
   TA-1535  SODIUM AZIDE             10 UG/PLATE             TA-1535  2-ANTHRAMINE  2.5 UG/PLATE
   TA-1537  9-AMINOACRIDINE          50 UG/PLATE             TA-1537  2-ANTHRAMINE  2.5 UG/PLATE
   TA-98    2-NITROFLUORENE          10 UG/PLATE             TA-98    2-ANTHRAMINE  2.5 UG/PLATE
   TA-100   SODIUM AZIDE             10 UG/PLATE             TA-100   2-ANTHRAMINE  2.5 UG/PLATE
   SOLVENT  50 UL/PLATE

   C INDICATES CONTAMINATION
```

VII. ASSAY ACCEPTANCE AND EVALUATION CRITERIA

Statistical methods are not currently used, and evaluation is based on the criteria included in this protocol.

Plate test data consists of direct revertant colony counts obtained from a set of selective agar plates seeded with populations of mutant cells suspended in a semisolid overlay. Because the test material and the cells are incubated in the overlay for approximately 2 days and a few cell divisions occur during the incubation period, the test is semiquantitative in nature. Although these features of the assay reduce the quantitation of results, they provide certain advantages not contained in a quantitative suspension test:

- The small number of cell divisions permits potential mutagens to act on replication DNA, which is often more sensitive than nonreplicating DNA.
- The combined incubation of the test article and the cells in the overlay permits constant exposure of the indicator cells for approximately 2 days.

A. Surviving Populations

Plate test procedures do not permit exact quantitation of the number of cells surviving chemical treatment. At low concentrations of the test material, the surviving population on the treatment plates is essentially the same as that on the negative control plate. At high concentrations, the surviving population is usually reduced by some fraction. Our protocol will normally employ several doses ranging over two or three log concentrations, the highest of these doses being selected to show slight toxicity as determined by subjective criteria.

B. Dose-Response Phenomena

The demonstration of dose-related increased in mutant counts is an important criterion in establishing metagenicity. A factor that might modify dose-response results for a mutagen would be the selection of doses that are too low (usually mutagenicity and toxicity are related). If the highest dose is far lower than a toxic concentration, no increases may be observed over the dose range selected. Conversely, if the lowest dose employed is highly cytotoxic, the test material may kill any mutants that are induced, and the test material will not appear to be mutagenic.

C. Control Tests

Positive and negative control assays were conducted with each experiment and consisted of direct-acting mutagens for nonactivation assays and mutagens that require metabolic biotransformation in activation assays.

Negative controls consisted of the test material solvent in the overlay agar together with the other essential components. The negative control plate for each strain gave a reference point to which the test data was compared. The positive control assay was conducted to demonstrate that the test systems were functional with known mutagens.

The following normal range of revertants for solvent controls are generally considered acceptable.

TA-1535: 8-30
TA-1537: 4-30
TA-98: 20-75
TA-100: 80-250

D. Evaluation Criteria for Ames Assay

Because the procedures to be used to evaluate the mutagenicity of the test material are semiquantitative, the criteria to be used to determine positive effects are inherently subjective and are based primarily on a historical data base. Most data sets will be evaluated using the following criteria.

1. Strains TA-1535 and TA-1537

If the solvent control value is within the normal range, a test material that produces a positive dose response over three concentrations with the highest increase equal to three times the solvent control value will be considered to be mutagenic.

2. Strains TA-98 and TA-100

If the solvent control value is within the normal range, a test material that produces a positive dose response over three concentrations with the highest increase equal to twice the solvent control value for TA-98 and TA-100 will be considered to be mutagenic.

3. Pattern

Because TA-1535 and TA-100 are both derived from the same parental strain (G-46), to some extent there is a built-in redundancy in the microbial assay. In general, the two strains of a set respond to the same mutagen and such a pattern is sought. Generally, if a strain responds to a mutagen in nonactivation tests, it will do so in activation tests.

4. Reproducibility

If a test material produces a response in a single test that cannot be reproduced in additional runs, the initial positive test data lose significance.

The preceding criteria are not absolute, and other extenuating factors may enter into a final evaluation decision. However, these criteria will be applied to the majority of situations and are presented to aid those individuals not familar with this procedure. As the data base is increased, the criteria for evaluation can be more firmly established.

E. Relation Between Mutagenicity and Carcinogenicity

It must be emphasized that the Ames *Salmonella*/Microsome Plate Assay is not a definitive test for chemical carcinogens. It is recognized, however, that correlative and functional relations have been demonstrated between these two endpoints. The results of comparative tests on 300 chemicals by McCann *et al*.[4] show an extremely good correlation between results of microbial mutagenesis tests and *in vivo* rodent carcinogenesis assays.

All evaluations and interpretation of the data to be presented in the final report will be based only on the demonstration, or lack, of mutagenic activity.

F. Criteria for Ranking Samples in the Ames Assay

The goal of EPA Level 1 Ames testing is to rank source streams by relative degree of genetic toxicity (mutagenicity). Samples are first identified as mutagenic or nonmutagenic by the criteria in Section D above and then ranked using the mutagenicity categories presented in the table below. The lowest concentration giving a positive response in any strain, with or without metabolic activation, is identified as the minimum effective concentration (MEC) for that sample. The mutagenicity of the sample is evaluated as high (H), moderate (M), low (L), or nondetectable (ND) according to the evaluation criteria developed in the Level 1 manual[1] and summarized below. Samples with no detectable activity at the maximum applicable dose (MAD) are ranked nondetectable (ND).

Ames Assay Mutagenicity Ranking Criteria[1]

Mutagenic Activity	Solids (MEC in μg/plate)	Liquids[a] (MEC in μl/plate)
High (H)	<50	<2
Moderate (M)	50-500	2-20
Low (L)	500-5000	20-200
Not Detectable (ND)	>5000	>200

[a] Concentration of organic extracts is based upon organic content (μg organics per plate) and not volume (μl extract per plate) of sample tested.

VIII. REFERENCES

1. Brusick, D.J., et al.: IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests. EPA Contract No. 68-02-2681, Technical Directive No. 501, Litton Bionetics, Inc., Kensington, MD, September 1980, 177 pp. In press.

2. Brusick, D.J.: Level 1 Bioassay Assessment and Data Formatting. EPA-600/7-80-079, Litton Bionetics Inc., Kensington, MD, April 1980, 100 pp.

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GENETICS ASSAY NO.: 5884
LBI SAFETY NO.: 7168

CYTOTOXIC EVALUATION OF A81-05-030-674 (EA-2 1+FILTER) IN THE RABBIT ALVEOLAR MACROPHAGE (RAM) CYTOTOXICITY ASSAY

FINAL REPORT

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LBI PROJECT NO.: 22064

REPORT DATE: NOVEMBER 1981

Litton BIONETICS

PREFACE

This assay conforms to the standard EPA Level 1 procedure for the rabbit alveolar macrophage (RAM) cytotoxicity assay as described in "IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests" (1). The data were evaluated and formatted as recommended in "Level 1 Biological Testing Assessment and Data Formatting" (2).

The RAM cytotoxicity assay has been shown to be a sensitive method for detecting cytotoxic activity for a variety of chemicals representing various chemical classes (3). This assay is one of several recommended by EPA to identify, categorize and rank the pollutant potential of influent and effluent streams from industrial and energy-producing processes. This assay has been well validated with a wide range of positive and negative control chemicals and complex environmental samples.

All procedures and documents pertaining to the receipt, storage, preparation, testing and evaluation of the test material shall conform to Litton Bionetics, Inc. standard operating procedures and the Good Laboratory Practices Regulations of 1979. Deviations from standard procedure shall be fully documented and noted in the report.

All test and control results in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Molecular Toxicology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20895. Copies of raw data will be supplied to the sponsor upon request.

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I. ASSAY SUMMARY

A. SPONSOR: Acurex Corporation

B. MATERIAL (TEST COMPOUND): GENETICS ASSAY NUMBER: 5884

1. Identification: A81-05-030-674 (EA-2 1+Filter)
2. Date Received: August 26, 1981
3. Physical Description: Fine, gray/black powder and fiberglass filter with embedded particulate material.

C. TYPE OF ASSAY: Rabbit Alveolar Macrophage (RAM) Cytotoxicity Assay

D. ASSAY DESIGN NUMBER: 443

E. STUDY DATES:

1. Initiation: October 1, 1981
2. Completion: October 14, 1981

F. SUPERVISORY PERSONNEL:

1. Study Director: Brian Myhr, Ph.D.
2. Laboratory Supervisor: Robert Young, M.S.

G. EVALUATION:

The combined particulate material from the filter and 1 micron catch caused a dose-related increase in toxicity for applied concentrations greater than approximately 20 µg/ml. All four assay parameters were responsive, but the primary effect was the reduction in cellular ATP content. The EC_{50} for the ATP content was 77 µg/ml, which resulted in an evaluation of moderate (M) toxicity for the combined particulate catch, using the toxicity categories defined for the IERL-EPA Level 1 RAM Cytotoxicity Assay.

Submitted by:

Study Director

Brian Myhr 11/24/81
Brian Myhr, Ph.D. Date
Associate Director,
Department of Molecular
Toxicology

11/24/81
David J. Brusick, Ph.D. Date
Director,
Department of Molecular
Toxicology

II. OBJECTIVE

The objective of this study was to determine and rank the cytotoxicity of A81-05-030-674 (EA-2 1+filter) to cultured rabbit alveolar macrophage (RAM) cells. The measure of cytotoxicity was the reduction in cell viability and adenosine triphosphate (ATP) content of the cultures after a 20 hour exposure to the test material. At the conclusion of the exposure period, the number of viable cells and total ATP content in the treated cultures were compared to the corresponding values in unexposed control cultures. The concentration of test material that reduced each experimental parameter by 50% was estimated graphically and referred to as the EC50 value. Standard EPA Level 1 toxicity evaluation criteria for the RAM cytotoxicity assay were used to rank the toxicity potential of the test material based upon the most sensitive parameter.

III. TEST MATERIAL

A. Description

The test material, as received, was comprised of two separate components. The first component, a fine black/gray powder, was the 1 μm SASS train particulate catch. The second component was a fiberglass filter with embedded particulate material. This dark material represented particulates less than 1 μm collected in the SASS train sample. Both components were supplied together in a Nalgene® screw-top bottle.

B. Handling and Preparation

The test material was received on August 26, 1981, and was assigned LBI assay number 5884 and LBI safety number 7168. The sample was stored at +4°C in the dark.

The filter portion of the sample required removal of the embedded particulates before testing could begin. The uncut filter was sonicated in cyclohexane as recommended by current IERL-EPA pretest sample preparation procedures[1]. The decanted particulate suspensions from three successive sonication treatments were combined and evaporated to dryness. The particulate residue was weighed and combined with the 1 μm particulate catch portion of the sample. A total of 264.42 mg of combined test material was available for testing and was comprised of 70.28 mg (26.6%) of <1 μm particulates removed from the filter and 194.14 mg (73.4%) of the 1 μm catch.

Approximately 34.4 mg of test material was used for the assay. The test material was suspended in serum-free EMEM culture medium at a concentration of 2000 μg/ml and incubated at 37°C on a roller drum for 8 hours. A fine suspension was formed that settled on standing. No pH changes were noted. The suspension was serially diluted with EMEM (serum-free) and applied to the cultures at a maximum concentration of 1000 μg/ml in the presence of 10% serum.

IV. MATERIALS

A. Indicator Cells

The assay employed short-term primary cultures of alveolar macrophage cells obtained by lung lavage of a male New Zealand white rabbit (2.25 kg). The rabbit was maintained on Purina Lab Rabbit Chow 5321 and water *ad libitum* and was examined for the absence of respiratory illnesses prior to use.

B. Media

The cells were maintained and treated in Eagle's Minimum Essential Medium (EMEM) with Earle's salts and supplemented with 10% fetal bovine serum (heat-inactivated), 100 units/ml penicillin, 100 μg/ml streptomycin, 17.6 μg/ml kanamycin, and 0.4 μg/ml amphotericin B.

C. Negative Controls

The negative control consisted of three untreated cultures carried through the same experimental time period as the treated cells. The average viability and ATP content of the negative control provided the reference points for determining the effects of different concentrations of the test material on the assay parameters.

V. EXPERIMENTAL DESIGN

A. Procurement of Cells

A rabbit was sacrificed by injection of Nembutal® (60 mg/ml) into the marginal ear vein, and sterile operating techniques were used to perform a tracheostomy. Prewarmed normal saline (30 ml) was then introduced into the lungs via a catheter and allowed to stand for 15 minutes. This lavage fluid was removed and placed into a 50-ml sterile centrifuge tube on ice. Nine additional lavages were similarly performed and collected, except the saline was removed shortly after its introduction into the lungs. Any lavage fluid containing blood or mucous was discarded. The lavages were centrifuged at 365 x g for 15 minutes and the cells resuspended in cold 0.85% saline. After two washes in saline by centrifugation, the cell pellets were resuspended in cold EMEM containing 20% serum and then combined. A cell count was obtained by hemocytometer and the suspension diluted to 5.02×10^5cells/ml. Viability was determined by trypan blue staining and the cells were not used if less than 95% viable. Also, a differential cell count from Wright-stained smears was performed to verify that the macrophage content was above 90%.

B. Sample Forms

The usual sample form for application to the cells is a suspension of particulate material. Solid samples are ground to fine particles and a weighed portion is suspended in a known volume of EMEM (0% FBS) for about eight hours to help leach any water-soluble material. Finely-divided test material may be suspended directly in culture medium without further grinding. Aqueous liquids, suspensions, or slurries containing less than 0.5% organic solvent are added by volume to culture medium.

Samples supplied as solutions in organic solvents are usually solvent-exchanged into DMSO before testing. Original sample volumes may first be reduced a maximum of 10-fold in a Kuderna-Danish concentrator, and the concentrative factor is used to convert assayed volumes into equivalent original sample volumes in the absence of information about solute concentration. An aliquot of the reduced volume is exchanged into DMSO by repeated, partial evaporation under a stream of nitrogen in a warm water bath (50°C); the evaporated volumes are replaced with equal volumes of DMSO.

Samples adsorbed on XAD-2 resin are extracted with methylene chloride or acetone in a Soxhlet apparatus for 24 hours. The extract is then concentrated and solvent-exchanged into DMSO. Alternatively, acetone extracts can be assayed directly at concentrations up to 2% by volume in the culture medium.

Samples impregnated on fiber glass or teflon filters are repeatedly sonicated in cyclohexane to remove particulates. The resulting cyclohexane particulate suspension is then evaporated to dryness and the particulates resuspended in EMEM culture medium at the desired concentration.

Sponsor-specified handling of sample materials will be followed if the above procedures are not applicable or a specific procedure is desired.

C. Dose Selection

Unless the approximate toxicity is already known or the sample size is limiting, the following usual dose ranges are tested for different sample forms. Dry, particulate material is tested at six dose levels from 1000 μg/ml to 3 μg/ml. Aqueous samples, suspensions, or slurries are tested from 600 μl to 3 μl/ml in six dose steps. Samples that are solvent-exchanged into DMSO are tested from 20 μl/ml (2% DMSO in growth medium) to 0.2 μl/ml, also in six dose steps. A second dose study is performed with an adjusted dose range if the EC50 was not located properly in the initial test. However, EC50 values greater than 1000 μl/ml for particulate material, 600 μl/ml for aqueous samples, or 20 μl/ml for organic solutions will not be determined.

This test material, A81-05-030-674 (EA-2 1+filter), was tested at 6 dose levels, starting at the maximum applicable dose (MAD) of 1000 μg/ml and including 600, 300, 100, 60 and 30 μg/ml.

D. Treatment

A series of 25 cm^2 culture flasks were prepared, each containing 2.0 ml of serum-free medium at 37°C and the test material at twice the desired final concentration. Three flasks were prepared for each test concentration. Aliquots of cell suspension (2 ml) were then added; each flask, therefore, contained 1×10^6 viable cells in a 4-ml volume of media containing 10% serum. The flasks were placed on a rocker platform in a 37°C incubator with a humidified atmosphere containing 5% CO_2. After sitting for about 30 minutes, the flasks were slowly rocked for the remainder of a 20-hour exposure period.

If the test substance causes a color change in the growth medium, the pH is determined in additional treated flasks. After the exposure period, the pH of the medium in the experimental flasks is again recorded.

E. Cell Viability Assay

At the end of the treatment period, the medium containing unattached cells was decanted into a centrifuge tube on ice. The attached cells were rinsed with 1 ml of 0.1% trypsin/0.01% versene and then incubated with 2 ml of the trypsin/versene solution for about 5 minutes at 37°C. The trypsinates and decanted media were combined for each culture to yield a 7-ml cell suspension for subsequent analysis.

A 1.0 ml aliquot of the cell suspension was removed for cell count and viability determination. The aliquot was combined with 1.0 ml of 0.4% trypan blue and counted by hemocytometer about 5 to 15 minutes later. The total number of cells counted per culture was the sum of the numbers found in five squares for each chamber of the hemocytometer (1 μl total volume). The numbers of live (colorless) and dead (blue) cells were recorded.

F. ATP Assay

ATP was immediately analyzed by extraction of a 0.1-ml sample of cell suspension with 0.9 ml of 90% DMSO. After 2 minutes at room temperature 5.0 ml cold MOPS buffer (0.01 M morpholinopropane sulfonic acid) at pH 7.4 was added and the extract mixed well and placed on ice. Aliquots of 10 µl were injected into a cuvette containing a luciferin-luciferase reaction mixture in a DuPont Model 760 Luminescence Biometer. The Biometer was calibrated daily with standard ATP solutions to provide a direct read-out of the ATP content. Each test sample was assayed at least twice to obtain repeatable readings.

VI. ASSAY ACCEPTANCE CRITERIA

The assay will be considered acceptable for evaluation of the test results if the following criteria are met:

1. The macrophage population is 90% or greater of the total nucleated cells collected by lung lavage.
2. The percent viability of the macrophages used to initiate the assay is 95% or greater.
3. The survival of viable macrophages in the negative control cultures over the 20 hour treatment priod is 70% or greater.
4. A sufficient number of data points (for five test concentrations or less) are available to clearly locate the EC50 of the most sensitive test parameter within a toxicity region as defined under Assay Evaluation Criteria.
5. The data points critical to the location of the EC50 for the most sensitive parameter are the averages of at least two treated cultures.
6. If all the test parameters yield EC50 values greater than 1000 µg/ml, 600 µl/ml for aqueous solutions, or 20 µl/ml for organic solutions, the plotted curves for ATP content and viability index parameters do not exceed 120% of the negative control.

VII. RESULTS

A. Interpretation

The test material was ingested by the macrophages and caused a decline in their viability when the applied concentration exceeded approximately 20 μg/ml. Absolute values for the assay parameters are given in Table 1, as well as the parameter values relative to the negative control cultures, and the relative values are plotted in Figures 1 and 2.

The most sensitive assay parameter was the culture ATP content, which yielded an EC_{50} of 77 μg/ml. This reduction in ATP was also reflected in the ATP/10^6 total cells parameter, which paralleled the culture ATP curve but was somewhat less sensitive (EC_{50} = 140 μg/ml). The ATP/10^6 cells measurement normally lags the ATP measurement because cellular disruption reduces the denominator of this parameter. In order for ATP/10^6 cells to be very responsive, the percent viability must decrease and the viability index (which measures the total number of viable cells) must not decrease as rapidly as the total ATP. As shown in Figure 1, the percent viability did decrease (EC_{50} just above the MAD of 1000 μg/ml) and the viability index declined with a more shallow slope than the ATP and leveled off near 40% of the negative control. Therefore, the primary effect of the combined particulate sample was to cause a drop in cellular ATP content and secondarily, a disruption of the macrophages. This toxicity was clearly evident in the low toxicity range of 100-1000 μg/ml, as defined for the IERL-EPA Level 1 RAM assay[1]. However, the inhibition began in the moderate region of 10-100 μg/ml for these parameters, and the ATP EC_{50} of 77 μg/ml resulted in an evaluation of moderate (M) toxicity for the test material. Although this response by the RAM cells closely approached the moderate/low toxicity borderline, the ATP EC_{50} would be expected to usually remain in the moderate region for repeated trials.

The macrophages collected for this assay had normal morphology and appeared to be in a healthy state. The initial viability was excellent (99.3%) and the survival of viable cells in the negative control was 96.0%. The average cellular ATP content of the negative controls was 25.1 x 10^8 fg ATP per 10^6 total cells which was within the historical range for acceptable cultures. These results achieved the assay acceptance criteria and provided confidence in the assumption that the collected data represented typical responses to the test material.

B. Tables and Figures

This report is based on the data provided in Table 1 and Figures 1 and 2.

TABLE 1
RABBIT ALVEOLAR MACROPHAGE (RAM) CYTOTOXICITY ASSAY DATA

LBI Assay No.: 5884

Test Material Identity: A81-05-030-674 (EA-2 1+Filter)

Test Date: October 13, 1981

Vehicle: EMEM

Initial Cell Viability: 99.3%

Viable Macrophage Seeded/Flask: 1.0 x 10^6 cells/flask

Macrophage Population Percentage: >90.0%

Survival of Negative Control
Macrophage Over Treatment Time: 96.0%

Sample	Concentration[a] µg/ml	Average Values per Culture Flask			ATP Per 10^6 Cells 10^8 fg	Viability %	Expressed as Percent of Negative Control			
		Viable Cells 10^6 Units	Total Cells 10^6 Units	ATP 10^8 fg[b]			Viability	Viability Index	ATP	ATP Per 10^6 Cells
NC[c]	---	0.97	1.01	25.4	25.1	96.0	100.0	100.0	100.0	100.0
TEST	30	0.85	0.91	18.7	20.5	93.4	97.3	87.6	73.6	81.7
TEST	60	0.67	0.72	14.7	20.4	93.1	97.0	69.1	57.9	81.3
TEST	100	0.60	0.68	10.7	15.7	88.2	91.9	61.9	42.1	62.5
TEST	300	0.44	0.58	3.7	6.4	75.9	79.1	45.4	14.6	25.5
TEST	600	0.44	0.70	2.7	3.9	62.9	65.5	45.4	10.6	15.5
TEST	1000	0.35	0.61	2.1	3.4	57.4	59.8	36.1	8.3	13.5
[d]EC50 VALUES: µg/ml:							>1000	210	77	140

Toxicity
Classification: Moderate

---

[a] pH change in culture medium: None observed

[b] fg = Femtogram (10^{-15} gram).

[c] NC = Negative Control, EMEM culture medium.

[d] Determined from data plots in Figures 1 and 2.

FIGURE 1

EC50 DETERMINATION FOR

PERCENT VIABILITY (O) AND VIABILITY INDEX (●)

A81-05-030-674

(EA-2 1+FILTER)

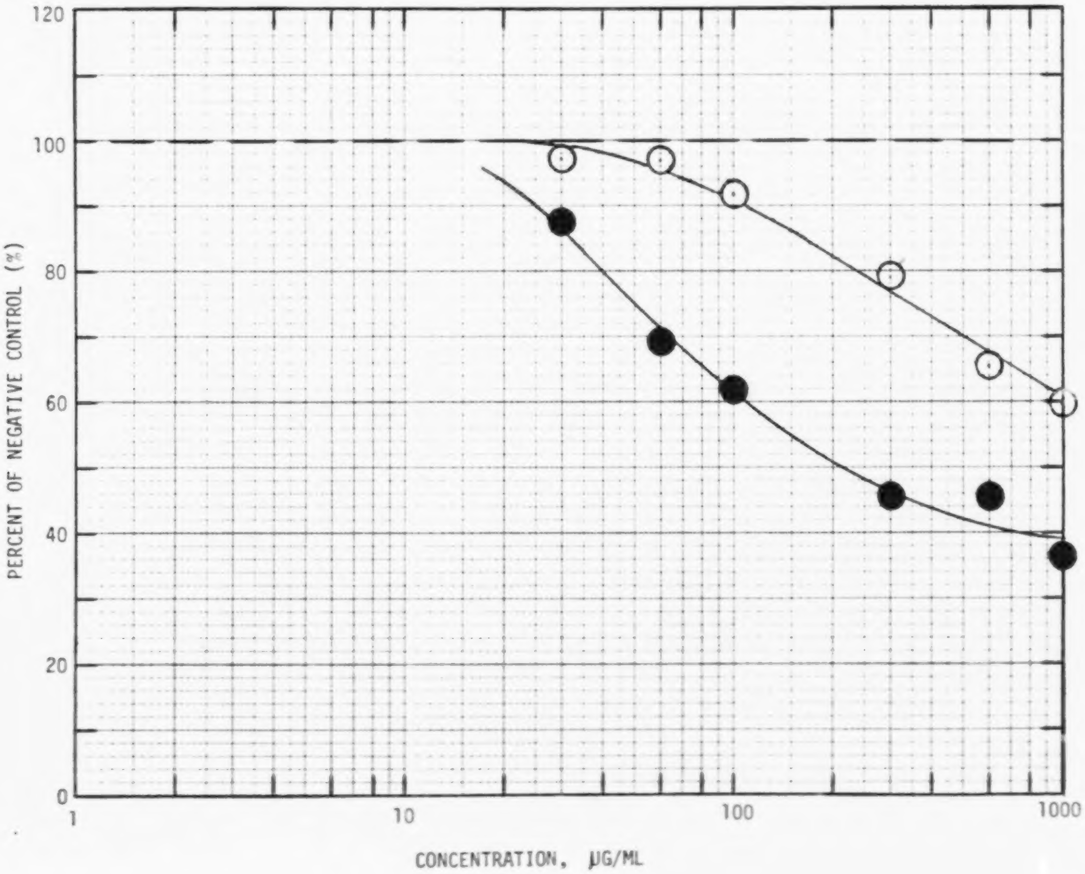


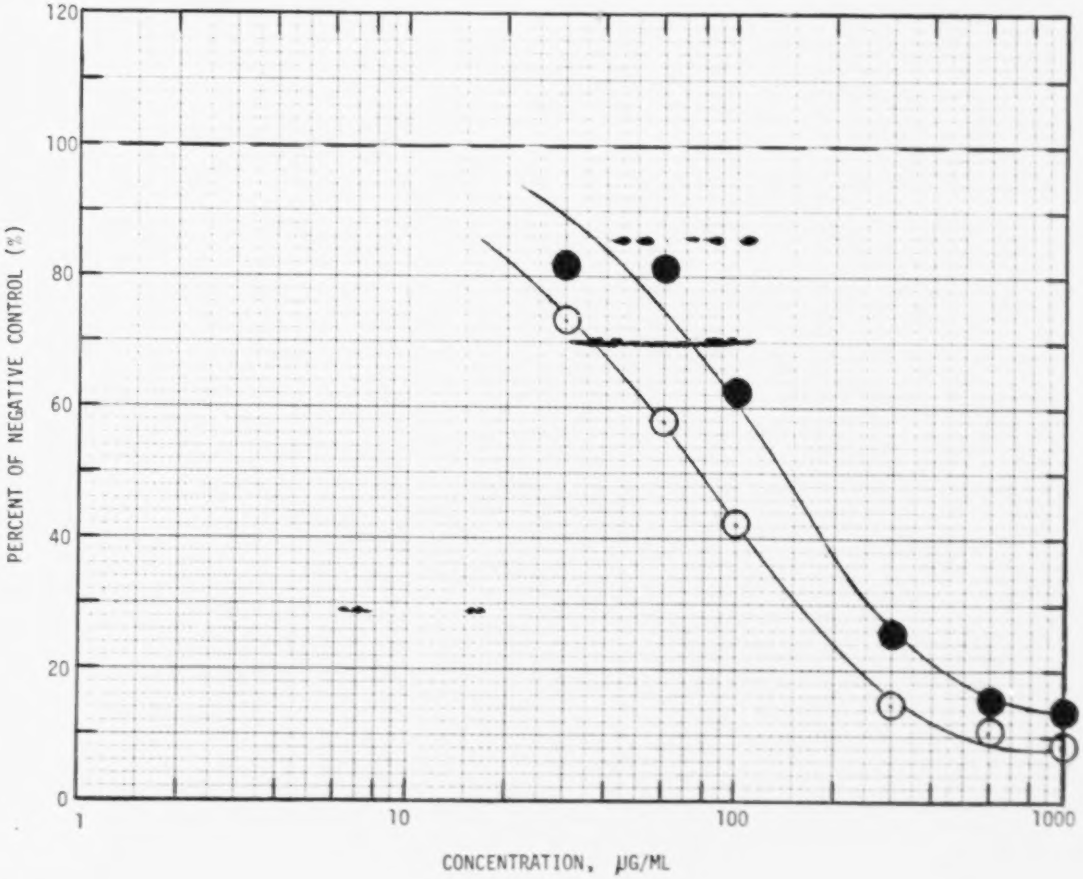
FIGURE 2

EC50 DETERMINATION FOR

ATP/FLASK (O) AND ATP/10^6 CELLS (●)

A81-05-030-674

(EA-2 1+FILTER)



VIII. ASSAY EVALUATION CRITERIA

The EC50 value represents the concentration of test material that reduces the most sensitive parameter of the RAM assay to 50% of the vehicle or negative control value. EC50 values are determined graphically by fitting a curve by eye through relative toxicity data plotted as a function of the logarithm of the applied concentration. Each data point normally represents the average of three culture dishes. Statistical analysis is unnecessary in most cases for evaluation.

The toxicity of the test material is evaluated as high, moderate, low, or nondetectable according to the range of EC50 values defined in the following table.

Toxicity[a]	Solids (EC_{50} in µg/ml)	Aqueous Liquids (EC_{50} in µl/ml)	Nonaqueous Liquids[b] (EC_{50} in µl/ml)
High	<10	<6	<0.2
Moderate	10 to 100	6 to 60	0.2-2
Low	100 to 1000	60 to 600	2-20
Not Detectable	>1000	>600	>20

[a] Evaluation criteria formulated by Litton Bionetics, Inc. for IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests[1].

[b] Criteria for nonaqueous liquids are tentative and under evaluation. If the organic or solid content is known, the solid evaluation criteria are applied.

Another evaluation scheme is proposed for extracts obtained from SASS train gas volumes. The proportion of the total gas volume corresponding to the volume of extract used in the bioassay is calculated and expressed as L/ml of culture medium (or DSCF/ml of culture medium). A criterion of 1000 L/ml is set as the limit for nondetectable toxicity. This gas volume corresponds to the average volume breathed by humans over a 2-hour period. The subsequent toxicity ranges are defined by 10-fold dilution steps to conform to standard procedure. The toxicity ranges are defined in the following table for liter and dry standard cubic feet units:

Toxicity	EC_{50} In Liters/ml (L/ml)	EC_{50} In Dry Standard Cubic Feet/ml (DSCF/ml)
High	<10	<0.35 DSCF
Moderate	10-100	0.35-3.5
Low	100-1000	3.5-35
Nondetectable	>1000	>35

IX. REFERENCES

1. Brusick, D.J., et al.: IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests. EPA Contract No. 68-02-2681, Technical Directive No. 501, Litton Bionetics, Inc., Kensington, MD, September 1980, 177 pp. In press.

2. Brusick, D.J.: Level 1 Bioassay Assessment and Data Formatting. EPA-600/7-80-079, Litton Bionetics, Inc., Kensington, MD, April 1980, 100 pp.

3. Brusick, D.J. and Young, R.R.: Level 1 Bioassay Sensitivity. EPA-600/7-81-135, Litton Bionetics, Inc., Kensington, MD, August 1981, pp. 52.

GENETICS ASSAY NO.: 5880
LBI SAFETY NO.: 7164

MUTAGENICITY EVALUATION OF
A81-05-030-676
(EA-2 XAD EXTRACT)
IN THE
EPA LEVEL 1
AMES SALMONELLA/MICROSOME
PLATE TEST

FINAL REPORT

SUBMITTED TO:

ACUREX CORPORATION
485 CLYDE AVENUE
MOUNTAIN VIEW, CALIFORNIA 94042

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20895

LBI PROJECT NO.: 22064

REPORT DATE: NOVEMBER 1981

Litton BIONETICS

PREFACE

This assay conforms to the standard EPA Level 1 procedure for the Ames Salmonella/microsome mutagenesis assay as described in "IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests"[1]. The data were evaluated and formatted as recommended in "Level 1 Biological Testing Assessment and Data Formatting"[2].

The Ames Salmonella/microsome mutagenesis assay has been shown to be a sensitive method for detecting mutagenic activity for a variety of chemicals representing various chemical classes[3]. This assay is one of several recommended by EPA to identify, categorize and rank the pollutant potential of influent and effluent streams from industrial and energy-producing processes. This assay has been well validated with a wide range of positive and negative control chemicals and complex environmental samples.

All procedures and documents pertaining to the receipt, storage, preparation, testing and evaluation of the test material shall conform to Litton Bionetics, Inc. standard operating procedures and the Good Laboratory Practices Regulations of 1979. Deviations from standard procedure shall be fully documented and noted in the report.

All test and control results in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Molecular Toxicology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20895. Copies of raw data will be supplied to the sponsor upon request.

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I. ASSAY SUMMARY

A. Sponsor: Acurex Corporation

B. Material (Test Compound): Genetics Assay Number: 5880

1. Identification: A81-05-030-676 (EA-2 XAD Extract)
2. Date Received: August 26, 1981
3. Physical Description: Clear, gold liquid.

C. Type of Assay: EPA Level 1 Ames Salmonella/Microsome Plate Test

D. Assay Design Number: 401 (EPA Level 1)

E. Study Dates:

1. Initiation: September 23, 1981
2. Completion: October 5, 1981

F. Supervisory Personnel:

A. Study Director: D.R. Jagannath, Ph.D.

G. Evaluation:

The test material, A81-05-030-676 (EA-2 XAD extract), contained 2.5 mg organics per ml after solvent exchange into dimethylsulfoxide (DMSO). The solvent exchanged sample was evaluated for its genetic activity in the EPA Level 1 Ames assay, directly and in the presence of a metabolic activation system. The test sample exhibited mutagenic activity with TA-98 and TA-100 in the presence and absence of S9 mix. The minimum effective concentration at which the mutagenic activity was observed was at 10 μl per plate (or 25 μg organics per plate) with TA-98 in the nonactivation assay. These tests indicate that the test material contains both frame shift and base-pair type mutagens. The mutagenic activity of the sample was classified as high (H) according to the IERL-EPA Level 1 evaluation criteria[1].

Submitted by:

Study Director

11/20/81

D.R. Jagannath, Ph.D. — Date
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Reviewed by:

[illegible]

David J. Brusick, Ph.D. — Date
Director,
Department of Molecular
Toxicology

II. OBJECTIVE

The objective of this study was to determine the genetic activity of A81-05-030-676 (EA-2 XAD extract) in the Salmonella/ microsome assay with and without the addition of mammalian metabolic activation preparations. The genetic activity of a sample is measured in these assays by its ability to revert the Salmonella indicator strains from histidine dependence to histidine independence. The degree of genetic activity of a sample is reflected in the number of revertants that are observed on the histidine-free medium.

III. TEST MATERIAL

A. Description

The test material was received as a clear, gold solution in methylene chloride. The sample contained 9.0 milligrams of organic material in an undetermined volume of methylene chloride. No information on the sampling parameters (such as the equivalent volume of stack gas represented by the sample) was provided.

B. Handling and Preparation

The test material was received at LBI on August 26, 1981. The sample was assigned LBI safety number 7164 and LBI assay number 5880. The sample was stored at +4°C in the dark.

Pretest sample preparation consisted of solvent exchanging the sample into dimethylsulfoxide (DMSO). The sample was transferred with methylene chloride rinses into a graduated conical tube. The methylene chloride was gradually evaporated (50°C under a stream of nitrogen) and DMSO was sequentially added. The sample was brought to volume in 3.6 ml of DMSO, giving a sample concentration of 2.5 mg organics per ml DMSO. The sample was transferred to a glass vial and sealed with a teflon-coated rubber septum.

Approximately 2.6 ml of test material was used for testing. Varying aliquots of the test material were added directly to the test mixtures to give the desired concentration.

IV. MATERIALS

A. Indicator Microorganisms

The Salmonella typhimurium strains used in this assay were obtained from Dr. Bruce Ames, University of California at Berkeley.[4-8] The following four strains were used.

Strain Designation	Gene Affected	Additional Mutations			Mutation Type Detected
		Repair	LPS	R Factor	
TA-1535	his G	Δ uvr B	rfa	-	Base-pair substitution
TA-1537	his C	Δ uvr B	rfa	-	Frameshift
TA-98	his D	Δ uvr B	rfa	pKM101	Frameshift
TA-100	his G	Δ uvr B	rfa	pKM101	Base-pair substitution

All the above strains have, in addition to the mutation in the histidine operon, mutation (rfa-) that leads to defective lipopolysaccharide coat, a deletion that covers genes involved in the synthesis of vitamin biotin (bio-) and in the repair of ultraviolet (uv) - induced DNA damage (uvrB-). The rfa- mutation makes the strains more permeable to many large molecules. The uvrB- mutation decreases repair of some types of chemically or physically damaged DNA and thereby enhances the strain's sensitivity to some mutagenic agents. The resistant transfer factor plasmid (R factor) pKM101 in TA-98 and TA-100 is believed to cause an increase in error-prone DNA repair that leads to many more mutations for a given dose of most mutagens.[8] In addition, plasmid pKM101 confers resistance to the antibiotic ampicillin, which is a convenient marker to detect the presence of plasmid in the cells.

All indicator strains are kept at 4°C on minimal medium plates supplemented with a trace of biotin and an excess of histidine. In addition, the plates with plasmid-carrying strains contain ampicillin (25 μg/ml) to ensure stable maintenance of plasmid pKM101. New stock culture plates are made as often as necessary from the frozen master cultures or from single colony reisolates that were checked for their genotypic characteristics (his, rfa uvrB, bio) and for the presence of plasmid. For each experiment, an inoculum from the stock culture plates is grown overnight at 37°C in nutrient broth (Oxoid CM67) and used.

B. Media

The bacterial strains were cultured in Oxoid Media #2 (Nutrient Broth). The selective medium was Vogen Bonner Medium E with 2% glucose.[10] The

overlay agar consisted of 0.6% purified agar with 0.05 mM histidine, 0.05 mM biotin and 0.1M NaCl according to the methods of Ames _et al_.[9]

C. Activation System

1. S9 Homogenate

A 9,000 x _g_ supernatant prepared from Sprague-Dawley adult male rat liver induced by Aroclor 1254 (Ames _et al_.[9]) was purchased commercially and used in these assays.

2. S9 Mix

S9 mix used in these assays consisted of the following components:

Components	Concentration per Milliliter S9 Mix
NADP (sodium salt)	4 µmoles
D-glucose-6-phosphate	5 µmoles
$MgCl_2$	8 µmoles
KCl	33 µmoles
Sodium phosphate buffer pH 7.4	100 µmoles
Organ homogenate from rat liver (S9 fraction)	100 µliters

V. EXPERIMENTAL DESIGN

A. Dosage Selection

Test strategy and dose selection depend upon sample type and sample availability. The Level 1 manual[1] recommends solids to be initially tested at the maximum applicable dose (MAD) of 5 mg per plate and at lower concentrations of 2.5, 1, 0.5, 0.1 and 0.05 mg per plate. Liquids are tested initially at the MAD of 200 µl per plate, and at lower concentrations of 100, 50 and 10 µl per plate. Samples are retested over a narrower range of concentrations with strains showing positive results initially. Alternate dose are employed if sample size is limiting or at the direction of the sponsor.

Doses selected to test this sample covered the recommended dose range for liquids. The highest dose was at the MAD level of 200 µl per plate and included three lower dose levels of 100, 50 and 10 µl per plate. These dose levels corresponded to 500, 250, 125, and 25 µg orgaincs per plate.

B. Mutagenicity Testing

The procedure used was based on the paper published by Ames *et. al.*[9] and was performed as follows:

1. Nonactivation Assay

To a sterile 13 x 100 mm test tube placed in a 43°C water bath the following was added in order:

- 2.00 ml of 0.6% agar containing 0.05 mM histidine and 0.05 mM biotin.
- 0.01 ml to 0.2 ml of a solution of the test chemical to give the appropriate dose.
- 0.1 ml to 0.2 ml of indicator organism(s).
- 0.50 ml of 0.2M phosphate buffer, pH 7.4.

This mixture was swirled gently and then poured onto minimal agar plates (see IV B, Media). After the top agar had set, the plates were incubated at 37°C for approximately 2 days. The number of *his+* revertant colonies growing on the plates were counted with an automatic colony counter and recorded.

2. Activation Assay

The activation assay was run concurrently with the nonactivation assay. The only difference was the addition of 0.5 ml of S9 mix (see IV C, Activation System) to the tubes in place of 0.5 ml of phosphate buffer which was added in nonactivation assays. All other details were similar to the procedure for nonactivation assays.

A detailed flow diagram for the plate incorporation assay is provided in Figure 1.

C. Control Compounds

A negative control consisting of the solvent used for the test material was also assayed concurrently with the test material. For negative controls, step 'b' of Nonactivation Assays was replaced by 0.05 ml of the solvent. The negative controls were employed for each indicator strain and were performed in the absence and presence of S9 mix. The solvent used to prepare the stock solution of the test material is given in the Results section of this report. All dilutions of the test material were made using this solvent. The amount of solvent used was equal to the maximum volume used to give the appropriate test dose.

Specific positive control compounds known to revert each strain were also used and assayed concurrently with the test material. The concentrations and specificities of these compounds to specific strains are given in the following table:

Assay	Chemical	Solvent	Concentration per plate (μg)	Salmonella Strains
Nonactivation	Sodium azide	Water	10.0	TA-1535, TA-100
	2-Nitrofluorene (NF)	Dimethylsulfoxide	10.0	TA-98
	9-aminoacridine (9AA)	Ethanol	50.0	TA-1537
Activation	2-anthramine (ANTH)	Dimethylsulfoxide	2.5	For all strains

D. Recording and Presenting Data

The number of colonies on each plate were counted and recorded on printed forms. These raw data were analyzed in a computer program and reported on a printout. The results are presented as revertants per plate for each indicator strain employed in the assay. The positive and solvent controls are provided as reference points.

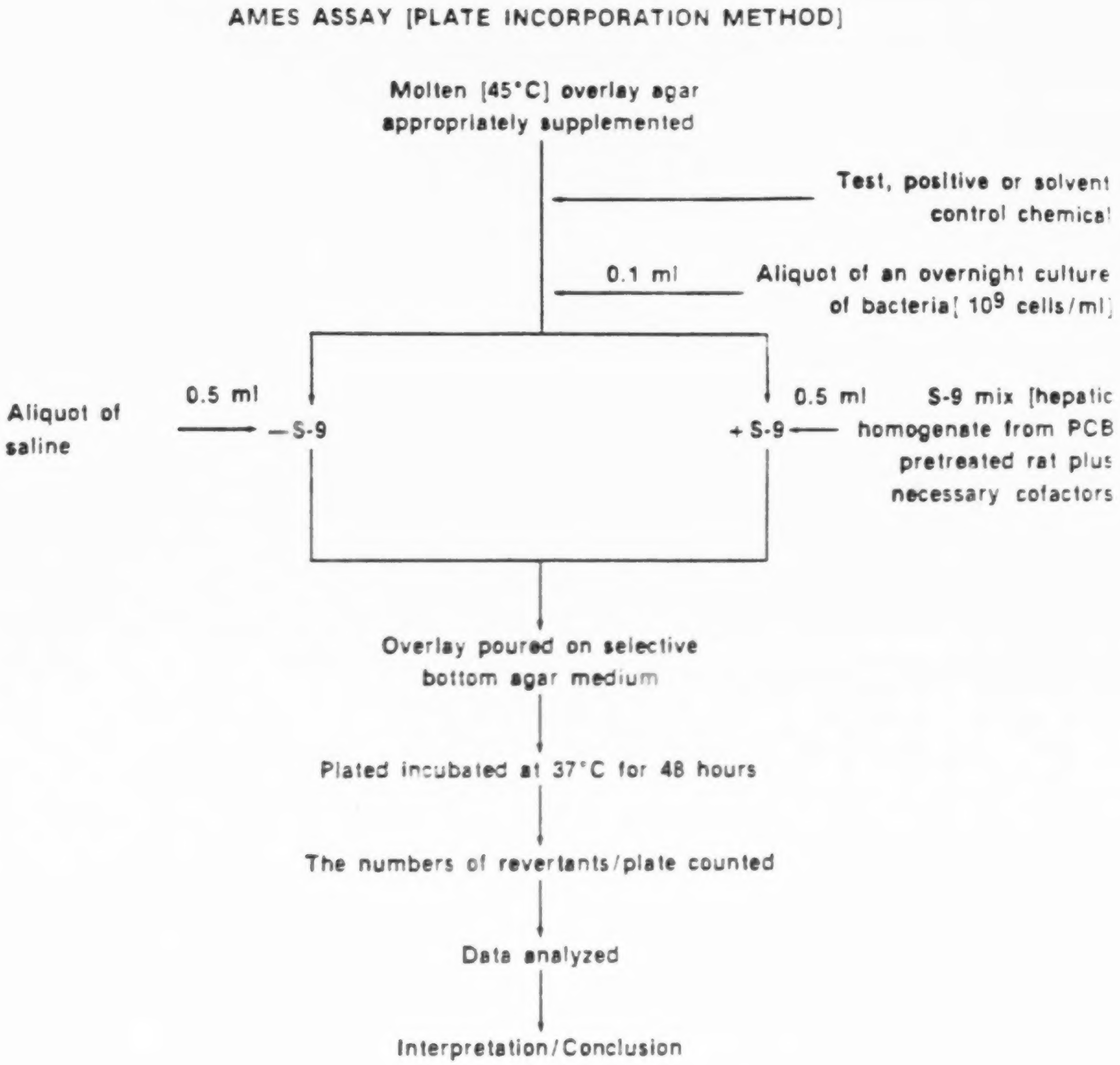
AMES ASSAY [PLATE INCORPORATION METHOD]
Molten [45°C] overlay agar appropriately supplemented
Test, positive or solvent control chemical
0.1 ml
Aliquot of an overnight culture of bacteria [10^9 cells/ml]
Aliquot of saline
0.5 ml
—S-9
+S-9
0.5 ml
S-9 mix [hepatic homogenate from PCB pretreated rat plus necessary cofactors
Overlay poured on selective bottom agar medium
Plated incubated at 37°C for 48 hours
The numbers of revertants/plate counted
Data analyzed
Interpretation/Conclusion

Figure 1 AMES SALMONELLA/MICROSOME MUTAGENESIS ASSAY

VI. RESULTS

A. Interpretations

The test material, A81-05-030-676 (EA-2 XAD extract), in methylene chloride was solvent exchanged to DMSO before conducting the EPA Level 1 Ames assays. The solvent exchanged material was tested directly and in the presence of liver microsomal enzymes from Aroclor induced rats. Due to the limited amount of the test sample, only TA-98 and TA-100 were used in the assays. Tests were conducted in duplicate except for TA-100 with activation, where only one plate per dose was used.

The results of the tests conducted on the sample in the absence of a metabolic activation were positive with both TA-98 and TA-100.

The results of the tests conducted on the sample in the presence of a rat liver activation system were positive with TA-98 and TA-100.

These results indicate that the test sample contains direct acting frame shift and base-pair type of mutagens. The minimum effective concentration (MEC) that exhibited mutagenic activity was at 10 μl per plate (or 25 μg organics per plate) with TA-98 in the nonactivation assays. This response was categorized as high (H) mutagenic activity using the IERL-EPA Level 1 evaluation critiera for the Ames Assay[1].

Solvent control and positive control values were within acceptable ranges. These results achieved assay acceptance criteria and provided confidence in the assumptions that the recorded data represented typical responses to the test material.

B. Tables

This report is based on the data provided in Table 1.

```
RESULTS                                               TABLE 1
--------------------------------

A.    NAME OR CODE DESIGNATION OF THE TEST COMPOUND:  A81-05-030-676 (EA-2 XAD EXTRACT)
B.    SOLVENT:  DMSO
C.    TEST INITIATION DATES:  10/01/81
D.    TEST COMPLETION DATE: 10/05/81
E.    S-9 LOT#: REF050
NOTE:   CONCENTRATIONS ARE GIVEN IN MICROLITERS   PER  PLATE

                                    R E V E R T A N T S   P E R   P L A T E
                                  ------------------------------------------------------------------------
TEST                SPECIES TISSUE   TA-98            TA-100
----                ------- ------ --------------- ---------------
                                      1    2    3     1    2    3
NONACTIVATION
-------------


SOLVENT CONTROL       ---     ---     30   30         134  128
POSITIVE CONTROL**    ---     ---    760  814        1192 1363

TEST COMPOUND
     10.00      UL    ---     ---     84   58         214  220
     50.00      UL    ---     ---    174  148         464  401
    100.00      UL    ---     ---    217  186         666  515
    200.00      UL    ---     ---    301  260         303  224

ACTIVATION
----------


SOLVENT CONTROL       RAT     LIVER   38   45         127  132
POSITIVE CONTROL***   RAT     LIVER 2036             2074 2145

TEST COMPOUND
     10.00      UL    RAT     LIVER   71   66         210    -
     50.00      UL    RAT     LIVER  228  172         332    -
    100.00      UL    RAT     LIVER  269  217         432    -
    200.00      UL    RAT     LIVER  366  274         444    -
------------------------------------------------------------------------------------------------------

**                                                          ***
   TA-98    2-NITROFLUORENE                   10 UG/PLATE      TA-98     2-ANTHRAMINE   2.5 UG/PLATE
   TA-100   SODIUM AZIDE                      10 UG/PLATE      TA-100    2-ANTHRAMINE   2.5 UG/PLATE
   SOLVENT  50 UL/PLATE
 - INDICATES TEST WAS NOT DONE
```

VII. ASSAY ACCEPTANCE AND EVALUATION CRITERIA

Statistical methods are not currently used, and evaluation is based on the criteria included in this protocol.

Plate test data consists of direct revertant colony counts obtained from a set of selective agar plates seeded with populations of mutant cells suspended in a semisolid overlay. Because the test material and the cells are incubated in the overlay for approximately 2 days and a few cell divisions occur during the incubation period, the test is semiquantitative in nature. Although these features of the assay reduce the quantitation of results, they provide certain advantages not contained in a quantitative suspension test:

- The small number of cell divisions permits potential mutagens to act on replication DNA, which is often more sensitive than nonreplicating DNA.
- The combined incubation of the test article and the cells in the overlay permits constant exposure of the indicator cells for approximately 2 days.

A. Surviving Populations

Plate test procedures do not permit exact quantitation of the number of cells surviving chemical treatment. At low concentrations of the test material, the surviving population on the treatment plates is essentially the same as that on the negative control plate. At high concentrations, the surviving population is usually reduced by some fraction. Our protocol will normally employ several doses ranging over two or three log concentrations, the highest of these doses being selected to show slight toxicity as determined by subjective criteria.

B. Dose-Response Phenomena

The demonstration of dose-related increased in mutant counts is an important criterion in establishing metagenicity. A factor that might modify dose-response results for a mutagen would be the selection of doses that are too low (usually mutagenicity and toxicity are related). If the highest dose is far lower than a toxic concentration, no increases may be observed over the dose range selected. Conversely, if the lowest dose employed is highly cytotoxic, the test material may kill any mutants that are induced, and the test material will not appear to be mutagenic.

C. Control Tests

Positive and negative control assays were conducted with each experiment and consisted of direct-acting mutagens for nonactivation assays and mutagens that require metabolic biotransformation in activation assays.

Negative controls consisted of the test material solvent in the overlay agar together with the other essential components. The negative control plate for each strain gave a reference point to which the test data was compared. The positive control assay was conducted to demonstrate that the test systems were functional with known mutagens.

The following normal range of revertants for solvent controls are generally considered acceptable.

TA-1535:	8-30
TA-1537:	4-30
TA-98:	20-75
TA-100:	80-250

D. Evaluation Criteria for Ames Assay

Because the procedures to be used to evaluate the mutagenicity of the test material are semiquantitative, the criteria to be used to determine positive effects are inherently subjective and are based primarily on a historical data base. Most data sets will be evaluated using the following criteria.

1. Strains TA-1535 and TA-1537

If the solvent control value is within the normal range, a test material that produces a positive dose response over three concentrations with the highest increase equal to three times the solvent control value will be considered to be mutagenic.

2. Strains TA-98 and TA-100

If the solvent control value is within the normal range, a test material that produces a positive dose response over three concentrations with the highest increase equal to twice the solvent control value for TA-98 and TA-100 will be considered to be mutagenic.

3. Pattern

Because TA-1535 and TA-100 are both derived from the same parental strain (G-46), to some extent there is a built-in redundancy in the microbial assay. In general, the two strains of a set respond to the same mutagen and such a pattern is sought. Generally, if a strain responds to a mutagen in nonactivation tests, it will do so in activation tests.

4. Reproducibility

If a test material produces a response in a single test that cannot be reproduced in additional runs, the initial positive test data lose significance.

The preceding criteria are not absolute, and other extenuating factors may enter into a final evaluation decision. However, these criteria will be applied to the majority of situations and are presented to aid those individuals not familar with this procedure. As the data base is increased, the criteria for evaluation can be more firmly established.

E. Relation Between Mutagenicity and Carcinogenicity

It must be emphasized that the Ames Salmonella/Microsome Plate Assay is not a definitive test for chemical carcinogens. It is recognized, however, that correlative and functional relations have been demonstrated between these two endpoints. The results of comparative tests on 300 chemicals by McCann *et al.*[4] show an extremely good correlation between results of microbial mutagenesis tests and *in vivo* rodent carcinogenesis assays.

All evaluations and interpretation of the data to be presented in the final report will be based only on the demonstration, or lack, of mutagenic activity.

F. Criteria for Ranking Samples in the Ames Assay

The goal of EPA Level 1 Ames testing is to rank source streams by relative degree of genetic toxicity (mutagenicity). Samples are first identified as mutagenic or nonmutagenic by the criteria in Section D above and then ranked using the mutagenicity categories presented in the table below. The lowest concentration giving a positive response in any strain, with or without metabolic activation, is identified as the minimum effective concentration (MEC) for that sample. The mutagenicity of the sample is evaluated as high (H), moderate (M), low (L), or nondetectable (ND) according to the evaluation criteria developed in the Level 1 manual[1] and summarized below. Samples with no detectable activity at the maximum applicable dose (MAD) are ranked nondetectable (ND).

Ames Assay Mutagenicity Ranking Criteria[1]

Mutagenic Activity	Solids (MEC in µg/plate)	Liquids[a] (MEC in µl/plate)
High (H)	<50	<2
Moderate (M)	50-500	2-20
Low (L)	500-5000	20-200
Not Detectable (ND)	>5000	>200

[a] Concentration of organic extracts is based upon organic content (µg organics per plate) and not volume (µl extract per plate) of sample tested.

VIII. REFERENCES

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GENETICS ASSAY NO.: 5880
LBI SAFETY NO.: 7164

CYTOTOXIC EVALUATION OF
A81-05-030-676
(EA-2 XAD EXTRACT)
IN THE
RODENT CELL (CHO)
CLONAL TOXICITY ASSAY

FINAL REPORT

SUBMITTED TO:

ACUREX CORPORATION
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MOUNTAIN VIEW, CALIFORNIA 94042

SUBMITTED BY:

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LBI PROJECT NO.: 22064

REPORT DATE: NOVEMBER 1981

Litton BIONETICS

PREFACE

This assay conforms to the standard EPA Level 1 procedure for the Chinese hamster ovary cell (CHO) clonal toxicity assay as described in "IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests" (1). The data were evaluated and formatted as recommended in "Level 1 Biological Testing Assessment and Data Formatting" (2).

The CHO clonal toxicity assay has been shown to be a sensitive method for detecting cytotoxic activity for a variety of chemicals representing various chemical classes (3). This assay is one of several recommended by EPA to identify, categorize and rank the pollutant potential of influent and effluent streams from industrial and energy-producing processes. This assay has been well validated with a wide range of positive and negative control chemicals and complex environmental samples.

All procedures and documents pertaining to the receipt, storage, preparation, testing and evaluation of the test material shall conform to Litton Bionetics, Inc. standard operating procedures and the Good Laboratory Practices Regulations of 1979. Deviations from standard procedure shall be fully documented and noted in the report.

All test and control results in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Molecular Toxicology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20895. Copies of raw data will be supplied to the sponsor upon request.

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I. ASSAY SUMMARY

A. SPONSOR: Acurex Corporation

B. MATERIAL (TEST COMPOUND): GENETICS ASSAY NUMBER: 5880

1. Identification: A81-05-030-676 (EA-2 XAD Extract)
2. Date Received: August 26, 1981
3. Physical Description: Clear, gold liquid

C. TYPE OF ASSAY: Rodent Cell (CHO) Clonal Toxicity Assay

D. ASSAY DESIGN NUMBER: 442

E. STUDY DATES:

1. Initiation: September 23, 1981
2. Completion: October 6, 1981

F. SUPERVISORY PERSONNEL:

1. Study Director: Brian C. Myhr, Ph.D.
2. Laboratory Supervisor: Robert Young, M.S.

G. EVALUATION:

The test material caused a slight increase in toxicity with increasing concentrations up to 1.0 μl/ml. The relative survival dropped to nearly zero at 3 μl/ml and was zero for doses of 6 μl/ml and above. The EC_{50} was estimated graphically to be 1.72 μl/ml which was equivalent to 4.3 μg of organics per ml. This sample was therefore evaluated to be in the high (H) toxicity category defined for the IERL-EPA Level 1 CHO clonal toxicity bioassay.[1]

Submitted by:

Study Director

Brian Myhr 11/20/81
Brian Myhr, Ph.D. Date
Associate Director,
Department of Molecular
Toxicology

Reviewed by:

11/[illegible]/81
David J. Brusick, Ph.D. Date
Director,
Department of Molecular
Toxicology

II. OBJECTIVE

The objective of this study was to determine and rank the cytotoxicity of A81-05-030-676 (EA-2 XAD extract) to cultured Chinese hamster cells (CHO-K1 cell line). The measure of cytotoxicity was the reduction in colony-forming ability after a 24-hour exposure to the test material. After a period of recovery and growth, the number of colonies that developed in the treated cultures was compared to the colony number in unexposed vehicle control cultures. The concentration of test material that reduced the colony number by 50% was estimated graphically and referred to as the EC50 value. Standard EPA Level 1 toxicity evaluation criteria for the CHO clonal toxicity assay were used to rank the toxicity potential of the test material.

III. TEST MATERIAL

A. Description

The test material was received as a clear, gold solution in methylene chloride. The sample contained 9.0 milligrams of organic material in 1.0 ml of methylene chloride. No information on the sampling parameters (such as the equivalent volume of stack gas represented by the sample) was provided.

B. Handling and Preparation

The test material was received at LBI on August 26, 1981. The sample was assigned LBI safety number 7164 and LBI assay number 5880. The sample was stored at +4°C in the dark.

Pretest sample preparation consisted of solvent-exchanging the sample into dimethylsulfoxide (DMSO). The sample was transferred with methylene chloride rinses into a graduated conical tube. The methylene chloride was gradually evaporated (50°C under a stream of nitrogen) and DMSO was sequentially added. The sample was brought to volume in 3.6 ml of DMSO, giving a sample concentration of 2.5 mg organics per ml DMSO. The sample was then transferred to a glass vial and sealed with a teflon-coated rubber septum.

A total volume of 0.45 ml of test sample was used in the CHO assay. The maximum concentration of 20 µl/ml was obtained by adding 0.12 ml of sample to 5.88 ml of F12 medium; this resulted in 2% (v/v) DMSO in the medium and effectively limited the concentration of test material that could be assayed. Only two plates were dosed at the top dose in order to conserve sample. Another 0.12 ml aliquot of sample was used to prepare the 10 µl/ml test concentration. An additional 0.21 ml of test sample was used to prepare a series of dilutions in DMSO from which 1:100 dilutions into growth medium were performed to obtain the lower assayed concentrations. Thus, except for the 20 µl/ml test concentration, the final DMSO concentration was constant at 1% (v/v).

IV. MATERIALS

A. Indicator Cells

The indicator cells for this study were Chinese hamster CHO-K1 cells (ATCC No. CCL 61) obtained from Flow Laboratories, Inc., Rockville, MD. This cell type was derived from ovarian tissue and has spontaneously transformed to a stable, hypodiploid line of rounded, fibroblastic cells with unlimited growth potential. Monolayer cultures have a fast doubling time of 11 to 14 hours, and untreated cells can normally be cloned with an efficiency of 80% or greater. Laboratory stock are maintained by routine serial subpassage. Cells are cultivated in Ham's F-12 nutrient medium at 37°C in 5 percent CO_2 with saturated humidity. Stocks are continually observed macroscopically and microscopically for possible microbial contamination. Laboratory cultures are periodically checked by culturing and staining methods for the absence of mycoplasma contamination. Laboratory cultures are discarded every three months and new cultures started from mycoplasma-free, long-term frozen cultures.

B. Media

The CHO-K1 cell line has an absolute requirement for proline and therefore must be maintained in culture medium containing sufficient amounts of this amino acid. Ham's F12 medium, which contains 3×10^{-4} M L-proline was used, supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 0.9 µl/ml of amphotericin B. A 10X formulation of Ham's F10 is available commercially and was used for the testing of aqueous test samples in order to avoid the dilution of medium components. This medium contains 1×10^{-4} L-proline and was supplemented in the same manner as F12, except that kanamycin at 40 µg/ml is included for additional protection against bacterial contamination. Both media formulations support the growth and cloning of CHO cells equally well.

C. Controls

The negative control consisted of three untreated cultures carried through the same experimental time period as the treated cells. Since the test material was tested as a solution in an organic vehicle (DMSO) and was diluted into the medium to provide each test concentration, two sets of vehicle control cultures containing the organic solvent at 1% and 2% by volume were prepared in triplicate.

The average number of colonies in the negative control established the cloning efficiency of the CHO cells used in the assay, and the appropriate vehicle control provided the reference points for determining the effects of different concentrations of the test material on cell survival.

V. EXPERIMENTAL DESIGN

A. Dose Selection

Unless the approximate toxicity is already known or the sample size is limiting, the following dose ranges are usually tested for different sample forms. Aqueous samples, suspensions, or slurries are tested from 600 µl/ml to 3 µl/ml, usually in six dose steps. Eight doses are often used when the amount of test sample is limited to provide a more precise description of toxicity in the event of sharp dose-response curves. Dry, particulate material is dissolved or suspended in DMSO, diluted into growth medium, and tested at six dose levels from 1000 µg/ml to 3 µg/ml. Samples that are solvent-exchanged into DMSO are tested from 20 µl/ml (2% DMSO in growth medium) to 0.2 µl/ml, also in six dose steps. A second dose study is performed with an adjusted dose range if the EC50 was not located properly in the initial test. However, EC50 values greater than 1000 µg/ml for particulate material, 600 µl/ml for aqueous samples, or 20 µl/ml for organic solutions will not be determined.

This sample, A81-05-030-676 (EA-2 XAD extract), was tested at eight dose levels. The concentrations started with the maximum applicable dose (MAD) of 20 µl/ml and included 10, 6, 3, 1, 0.6, 0.3, and 0.1 µl/ml. The corresponding concentration of organics at the MAD level was 50 µg/ml; the lower doses were equivalent to 25, 15, 7.5, 2.5, 1.5, 0.75, and 0.25 µg organics/ml.

B. Clonal Toxicity Assay

Cells from monolayer stock cultures in logarithmic growth phase were trypsinized with 0.1% trypsin plus 0.01% versene for 4 minutes and the density of the resulting cell suspension determined by hemocytometer. A number of 60-mm culture dishes were then seeded with 200 cells and 4 ml of culture medium per dish. The cultures were incubated for approximately 6 hours at 37°C in a humidified atmosphere containing 5% CO_2 to allow attachment of the cells. The 6-hour attachment period was used in order to avoid cell division and the subsequent formation of two-cell colonies prior to treatment.

The medium was aspirated from the cultures and 4 ml medium containing the test material applied. Three cultures were exposed to each test concentration. After an exposure time of 24 hours at 37°C, the medium was removed by aspiration and each culture washed three times with approximately 4 ml aliquots of Dulbecco's phosphate buffered saline (pre-warmed to 37°C). Fresh culture medium (5 ml) was placed in each dish and incubation at 37°C is continued for an additional 6 days to allow colony development.

The test material caused a color change in the culture medium, the pH of the medium containing the high dose would be determined at the time of treatment. The pH at the lowest dose that results in a slight color change would also recorded. At the end of the treatment period, the pH values of the discarded media from the two described treatments would be recorded again. No sample related pH effects were noted.

After the incubation period, the medium was drained from the cultures and the surviving colonies fixed with 100% ethanol and stained with Giemsa. Colonies were counted by eye; tiny colonies of approximately 50 cells or less were arbitrarily excluded from the counts.

VI. ASSAY ACCEPTANCE CRITERIA

The assay is considered acceptable for evaluation of the test results if the following criteria are met:

- The average cloning efficiency of the CHO-K1 cells in the negative controls is 70% or greater, but not exceeding 115%.
- The distribution of colonies in the treated cultures is generally uniform over the surface of the culture dish.
- The data points for each test concentration critical to the location of the EC50 are the averages of at least two treated cultures.
- A sufficient number of test concentrations are available to clearly locate the EC50 within a toxicity region as defined under Assay Evaluation Criteria.
- If the EC50 value is greater than 1000 µg/ml, 600 µliters of aqueous sample/ml, or 20 µliters of nonaqueous sample/ml, the plotted curve does not exceed 110% of the negative control.

VII. RESULTS

A. Interpretation

The application of sample A81-05-030-676 (EA-2 XAD extract) to the CHO cell cultures caused a rapid lowering of the number of cells able to form colonies as the concentration increased above 1.0 µl/ml. Relative survival values were calculated as the ratio of colonies formed in treated cultures to the colonies formed in the appropriate vehicle control, and these relative survival values were plotted against the concentration of test material. As shown in Figure 1, the relative survival decreased gradually in the 0.1 to 1.0 µl/ml range and dropped to nearly zero at the 3.0 µl/ml dose level.

The concentration expected to kill 50 percent of the cells (EC_{50}) was found to be 1.72 µl of test material per ml of culture medium. This concentration was equivalent to 4.3 µg of organic material per ml of culture medium. This value placed the test material in high (H) toxicity range defined for the IERL-EPA CHO clonal toxicity bioassay.

The cells used for the assay were in logarithmic growth phase and were 98.9 percent viable. About 89 percent of the seeded cells formed colonies in the negative control. Colony growth was normal and well distributed on the culture dishes. The combined results were considered to achieve assay acceptance criteria and provided confidence in the assumption that the recorded data represented typical responses to the test material.

B. Tables and Figures

This report is based on the data provided in Table 1 and Figure 1.

TABLE 1
RODENT CELL (CHO) CLONAL TOXICITY ASSAY

Sample Identity: A81-05-030-676 (EA-2 XAD Extract)

Description of Sample: Clear, gold liquid

LBI Assay No.: 5880

Date Received: August 26, 1981

Test Date: September 29, 1981

Vehicle: DMSO

Cell Type: CHO-K1

Cells Seeded per Dish: 200

EC50 Value: 1.72 µl/ml (4.3 µg organics/ml)

Toxicity Classification: High (H)

pH Alterations: None

Comments on Treatment: Sample prepared in DMSO at a concentration of 2.5 µg organics/µl

COLONY COUNTS

Sample	Applied Concentration µl/ml	Dish #1	Dish #2	Dish #3	Average Count	Relative Survival[a] %	Cloning Efficiency %
NC[b]	---	170	183	178	177.0	---	88.5
VC, 1%[c]	10	157	158	164	159.7	100.0	79.9
VC, 2%	20	146	153	137	145.3	100.0	72.7
TEST	0.1	145	168	158	157.0	98.3	
TEST	0.3	136	153	157	148.7	93.1	
TEST	0.6	125	134	140	133.0	83.3	
TEST	1.0	133	132	132	132.3	82.8	
TEST	3.0	0	0	3	1.0	0.6	
TEST	6.0	0	0	0	0	0	
TEST	10.0	0	0	0	0	0	
TEST	20.0	0	0	S[d]	0	0	

---

[a] Relative to 2% VC for 20 µl/ml treatment and to 1% VC for other treatments.
[b] NC = Negative Control, F_{12} medium
[c] VC = Vehicle Control, percent DMSO given
[d] S = Plate not set up to conserve limited test sample.

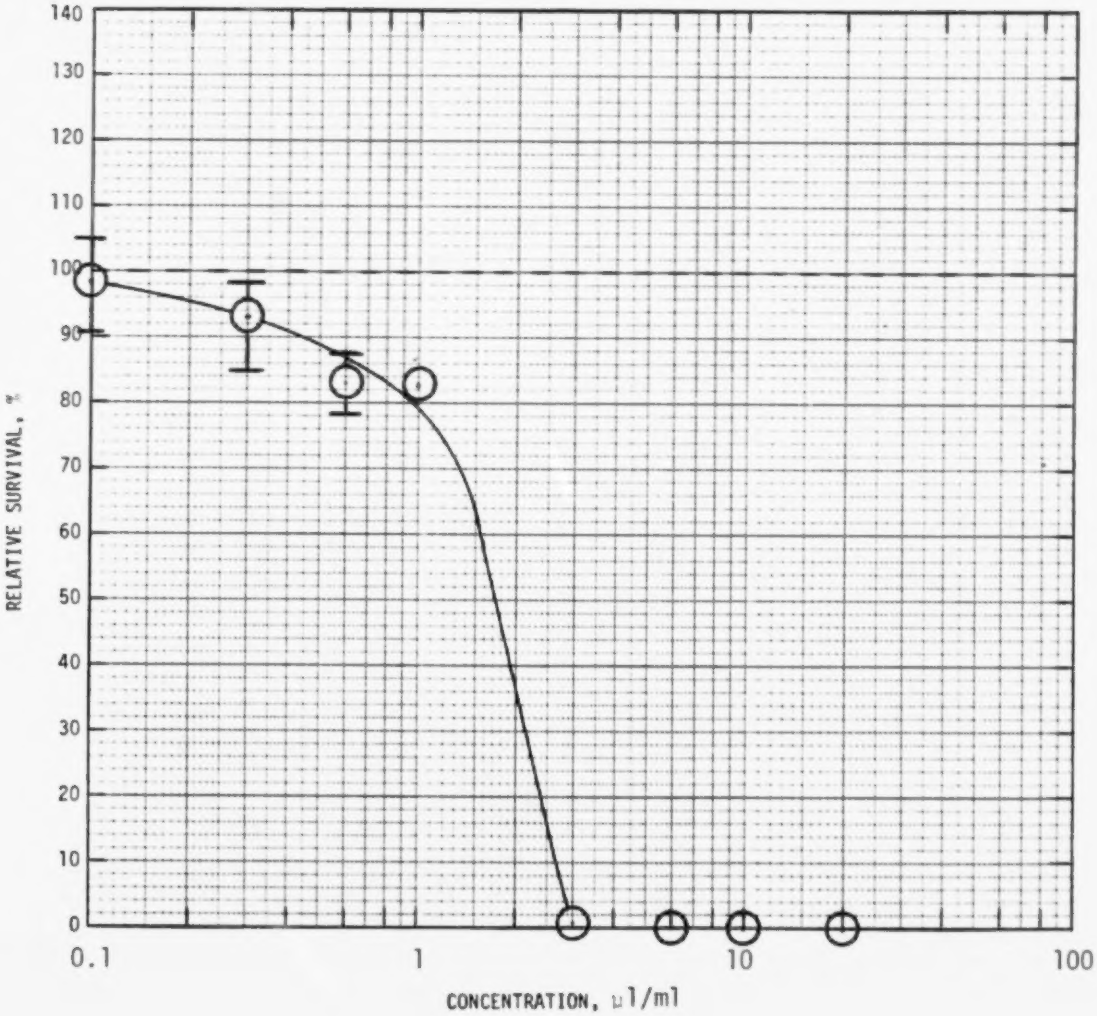
FIGURE 1

RODENT CELL (CHO) CLONAL TOXICITY ASSAY

EC_{50} DETERMINATION

A81-05-030-676

(EA-2 XAD EXTRACT)



VIII. ASSAY EVALUATION CRITERIA

The EC50 value represents the concentrations of test material that reduces the colony-forming ability of CHO cells to 50% of the vehicle or negative control value. EC50 values are determined graphically by fitting a curve by eye through relative survival data plotted as a function of the logarithm of the applied concentration. Each data point normally represents the average of three culture dishes. In order to indicate the variability of the data, the high and low colony counts for each concentration are used to calculate the relative survivals, and the range is shown by a bar at the position of the plotted average. If no bar is shown, the variability was within the size of the symbol. Statistical analysis is unnecessary in most cases for evaluation.

The toxicity of the test material is evaluated as high, moderate, low, or nondetectable according to the range of EC50 values defined in the following table.

Toxicity[a]	Solids (EC_{50} in µg/ml)	Aqueous Liquids (EC_{50} in µl/ml)	Nonaqueous Liquids[b] (EC_{50} in µl/ml)
High	<10	<6	<0.2
Moderate	10 to 100	6 to 60	0.2-2
Low	100 to 1000	60 to 600	2-20
Not Detectable	>1000	>600	>20

[a] Evaluation criteria formulated by Litton Bionetics, Inc. for IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests.

[b] Criteria for nonaqueous liquids are tentative and under evaluation. If the organic or solids content is known, the sample is evaluated under the solids criteria.

Another evaluation scheme is proposed for extracts obtained from SASS train gas volumes. The proportion of the total gas volume corresponding to the volume of extract used in the bioassay is calculated and expressed as L/ml of culture medium (or DSCF/ml of culture medium). A criterion of 1000 L/ml is set as the limit for nondetectable toxicity. This gas volume corresponds to the average volume breathed by humans over a 2-hour period. The subsequent toxicity ranges are defined by 10-fold dilution steps to conform to standard procedure. The toxicity ranges are defined in the following table for liter and dry standard cubic feet units:

Toxicity	EC_{50} In Liters/ml (L/ml)	EC_{50} In Dry Standard Cubic Feet/ml (DSCF/ml)
High	<10	<0.35 DSCF
Moderate	10-100	0.35-3.5
Low	100-1000	3.5-35
Nondetectable	>1000	>35

IX. REFERENCES

1. Brusick, D.J., et al.: IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests. EPA Contract No. 68-02-2681, Technical Directive No. 501, Litton Bionetics, Inc., Kensington, MD, September 1980, 177 pp. In press.

2. Brusick, D.J.: Level 1 Bioassay Assessment and Data Formatting. EPA-600/7-80-079, Litton Bionetics, Inc., Kensington, MD, April 1980, 100 pp.

3. Brusick, D.J. and Young, R.R.: Level 1 Bioassay Sensitivity. EPA-600/7-81-135, Litton Bionetics, Inc., Kensington, MD, August 1981, pp 52.

GENETICS ASSAY NO.: 5887
LBI SAFETY NO.: 7171

MUTAGENICITY EVALUATION OF A81-05-030-744 (EA-2 FLYASH) IN THE EPA LEVEL 1 AMES SALMONELLA/MICROSOME PLATE TEST

FINAL REPORT

SUBMITTED TO:

ACUREX CORPORATION
485 CLYDE AVENUE
MOUNTAIN VIEW, CALIFORNIA 94042

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
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LBI PROJECT NO.: 22064

REPORT DATE: NOVEMBER 1981

Litton BIONETICS

PREFACE

This assay conforms to the standard EPA Level 1 procedure for the Ames Salmonella/microsome mutagenesis assay as described in "IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests"[1]. The data were evaluated and formatted as recommended in "Level 1 Biological Testing Assessment and Data Formatting"[2].

The Ames Salmonella/microsome mutagenesis assay has been shown to be a sensitive method for detecting mutagenic activity for a variety of chemicals representing various chemical classes[3]. This assay is one of several recommended by EPA to identify, categorize and rank the pollutant potential of influent and effluent streams from industrial and energy-producing processes. This assay has been well validated with a wide range of positive and negative control chemicals and complex environmental samples.

All procedures and documents pertaining to the receipt, storage, preparation, testing and evaluation of the test material shall conform to Litton Bionetics, Inc. standard operating procedures and the Good Laboratory Practices Regulations of 1979. Deviations from standard procedure shall be fully documented and noted in the report.

All test and control results in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Molecular Toxicology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20895. Copies of raw data will be supplied to the sponsor upon request.

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I. ASSAY SUMMARY

A. Sponsor: Acurex Corporation

B. Material (Test Compound): Genetics Assay Number: 5887

1. Identification: A81-05-030-744 (EA-2 Flyash)
2. Date Received: August 26, 1981
3. Physical Description: Gray and white particles with larger (long and thin) black chunks.

C. Type of Assay: EPA Level 1 Ames Salmonella/Microsome Plate Test

D. Assay Design Number: 401 (EPA Level 1)

E. Study Dates:

1. Initiation: September 23, 1981
2. Completion: September 28, 1981

F. Supervisory Personnel:

A. Study Director: D.R. Jagannath, Ph.D.

G. Evaluation:

The test material, A81-05-030-744 (EA-2 flyash), was tested for activity in the Ames *Salmonella* mutagenicity assay over a concentration range of 0.05 mg/plate to 5.0 mg/plate. The test was performed in duplicate under nonactivation and activation test conditions with strains TA-1535, TA-1537, TA-98, and TA-100.

The sample was not mutagenic under the test conditions employed and was ranked as having nondetectable (ND) mutagenic activity as defined by the IERL-EPA Level 1 criteria for the Ames bioassay[1].

Submitted by:

Study Director

D R Jagannath 11/24/81

D.R. Jagannath, Ph.D. Date
Section Chief,
Submammalian Genetics,
Department of Molecular
Toxicology

Reviewed by:

David Brusick 11/24/81

David J. Brusick, Ph.D. Date
Director,
Department of Molecular
Toxicology

II. OBJECTIVE

The objective of this study was to determine the genetic activity of A81-05-030-744 (EA-2 flyash) in the Salmonella/ microsome assay with and without the addition of mammalian metabolic activation preparations. The genetic activity of a sample is measured in these assays by its ability to revert the Salmonella indicator strains from histidine dependence to histidine independence. The degree of genetic activity of a sample is reflected in the number of revertants that are observed on the histidine-free medium.

III. TEST MATERIAL

A. Description

The test material was received as gray and white particles with larger black chunks (15 gm) and was used without further preparation. No information on actual particle size distribution or on sampling parameters was received.

B. Handling and Preparation

The test material was received at LBI on August 26, 1981. The sample was assigned LBI safety number 7171 and LBI assay number 5887. The sample was stored at +4°C in the dark.

A total of 242.89 mg of test material was weighed and suspended in 24.3 ml of dimethylsulfoxide. The sample formed an opaque suspension after vortexing that settled upon standing. The suspension was incubated at 37°C on a shaker overnight to help leach material out of the particulates. Serial dilutions were made in DMSO such that 50 μl aliquots of each dilution give the desired concentration. The suspension was well mixed when aliquots were removed for dosing.

IV. MATERIALS

A. Indicator Microorganisms

The *Salmonella typhimurium* strains used in this assay were obtained from Dr. Bruce Ames, University of California at Berkeley.[4-8] The following four strains were used.

Strain Designation	Gene Affected	Additional Mutations			Mutation Type Detected
		Repair	LPS	R Factor	
TA-1535	*his* G	Δ *uvr* B	*rfa*	-	Base-pair substitution
TA-1537	*his* C	Δ *uvr* B	*rfa*	-	Frameshift
TA-98	*his* D	Δ *uvr* B	*rfa*	pKM101	Frameshift
TA-100	*his* G	Δ *uvr* B	*rfa*	pKM101	Base-pair substitution

All the above strains have, in addition to the mutation in the histidine operon, mutation (*rfa*-) that leads to defective lipopolysaccharide coat, a deletion that covers genes involved in the synthesis of vitamin biotin (*bio*-) and in the repair of ultraviolet (uv) - induced DNA damage (*uvr*B-). The *rfa*- mutation makes the strains more permeable to many large molecules. The *uvr*B- mutation decreases repair of some types of chemically or physically damaged DNA and thereby enhances the strain's sensitivity to some mutagenic agents. The resistant transfer factor plasmid (R factor) pKM101 in TA-98 and TA-100 is believed to cause an increase in error-prone DNA repair that leads to many more mutations for a given dose of most mutagens.[8] In addition, plasmid pKM101 confers resistance to the antibiotic ampicillin, which is a convenient marker to detect the presence of plasmid in the cells.

All indicator strains are kept at 4°C on minimal medium plates supplemented with a trace of biotin and an excess of histidine. In addition, the plates with plasmid-carrying strains contain ampicillin (25 μg/ml) to ensure stable maintenance of plasmid pKM101. New stock culture plates are made as often as necessary from the frozen master cultures or from single colony reisolates that were checked for their genotypic characteristics (*his*, *rfa* *uvr*B, *bio*) and for the presence of plasmid. For each experiment, an inoculum from the stock culture plates is grown overnight at 37°C in nutrient broth (Oxoid CM67) and used.

B. Media

The bacterial strains were cultured in Oxoid Media #2 (Nutrient Broth). The selective medium was Vogen Bonner Medium E with 2% glucose.[10] The

overlay agar consisted of 0.6% purified agar with 0.05 mM histidine, 0.05 mM biotin and 0.1M NaCl according to the methods of Ames _et al._[9]

C. Activation System

1. S9 Homogenate

A 9,000 x _g_ supernatant prepared from Sprague-Dawley adult male rat liver induced by Aroclor 1254 (Ames _et al._[9]) was purchased commercially and used in these assays.

2. S9 Mix

S9 mix used in these assays consisted of the following components:

Components	Concentration per Milliliter S9 Mix
NADP (sodium salt)	4 μmoles
D-glucose-6-phosphate	5 μmoles
$MgCl_2$	8 μmoles
KCl	33 μmoles
Sodium phosphate buffer pH 7.4	100 μmoles
Organ homogenate from rat liver (S9 fraction)	100 μliters

V. EXPERIMENTAL DESIGN

A. Dosage Selection

Test strategy and dose selection depend upon sample type and sample availability. The Level 1 manual[1] recommends solids to be initially tested at the maximum applicable dose (MAD) of 5 mg per plate and at lower concentrations of 2.5, 1, 0.5, 0.1 and 0.05 mg per plate. Liquids are tested initially at the MAD of 200 µl per plate, and at lower concentrations of 100, 50 and 10 µl per plate. Samples are retested over a narrower range of concentrations with strains showing positive results initially. Alternate dose are employed if sample size is limiting or at the direction of the sponsor.

Doses selected to test this sample covered the recommended dose range for solids. The highest dose was at the MAD level of 5 mg per plate and included five lower dose levels of 2.5, 1, 0.5, 0.1, and 0.05 mg per plate.

B. Mutagenicity Testing

The procedure used was based on the paper published by Ames et. al.[9] and was performed as follows:

1. Nonactivation Assay

To a sterile 13 x 100 mm test tube placed in a 43°C water bath the following was added in order:

- 2.00 ml of 0.6% agar containing 0.05 mM histidine and 0.05 mM biotin.
- 0.05 ml of a suspension of the test chemical to give the appropriate dose.
- 0.1 ml to 0.2 ml of indicator organism(s).
- 0.50 ml of 0.2M phosphate buffer, pH 7.4.

This mixture was swirled gently and then poured onto minimal agar plates (see IV B, Media). After the top agar had set, the plates were incubated at 37°C for approximately 2 days. The number of his+ revertant colonies growing on the plates were counted with an automatic colony counter and recorded.

2. Activation Assay

The activation assay was run concurrently with the nonactivation assay. The only difference was the addition of 0.5 ml of S9 mix (see IV C, Activation System) to the tubes in place of 0.5 ml of phosphate buffer which was added in nonactivation assays. All other details were similar to the procedure for nonactivation assays.

A detailed flow diagram for the plate incorporation assay is provided in Figure 1.

C. Control Compounds

A negative control consisting of the solvent used for the test material was also assayed concurrently with the test material. For negative controls, step 'b' of Nonactivation Assays was replaced by 0.05 ml of the solvent. The negative controls were employed for each indicator strain and were performed in the absence and presence of S9 mix. The solvent used to prepare the stock solution of the test material is given in the Results section of this report. All dilutions of the test material were made using this solvent. The amount of solvent used was equal to the maximum volume used to give the appropriate test dose.

Specific positive control compounds known to revert each strain were also used and assayed concurrently with the test material. The concentrations and specificities of these compounds to specific strains are given in the following table:

Assay	Chemical	Solvent	Concentration per plate (µg)	*Salmonella* Strains
Nonactivation	Sodium azide	Water	10.0	TA-1535, TA-100
	2-Nitrofluorene (NF)	Dimethyl-sulfoxide	10.0	TA-98
	9-aminoacridine (9AA)	Ethanol	50.0	TA-1537
Activation	2-anthramine (ANTH)	Dimethyl-sulfoxide	2.5	For all strains

D. Recording and Presenting Data

The number of colonies on each plate were counted and recorded on printed forms. These raw data were analyzed in a computer program and reported on a printout. The results are presented as revertants per plate for each indicator strain employed in the assay. The positive and solvent controls are provided as reference points.

AMES ASSAY [PLATE INCORPORATION METHOD]

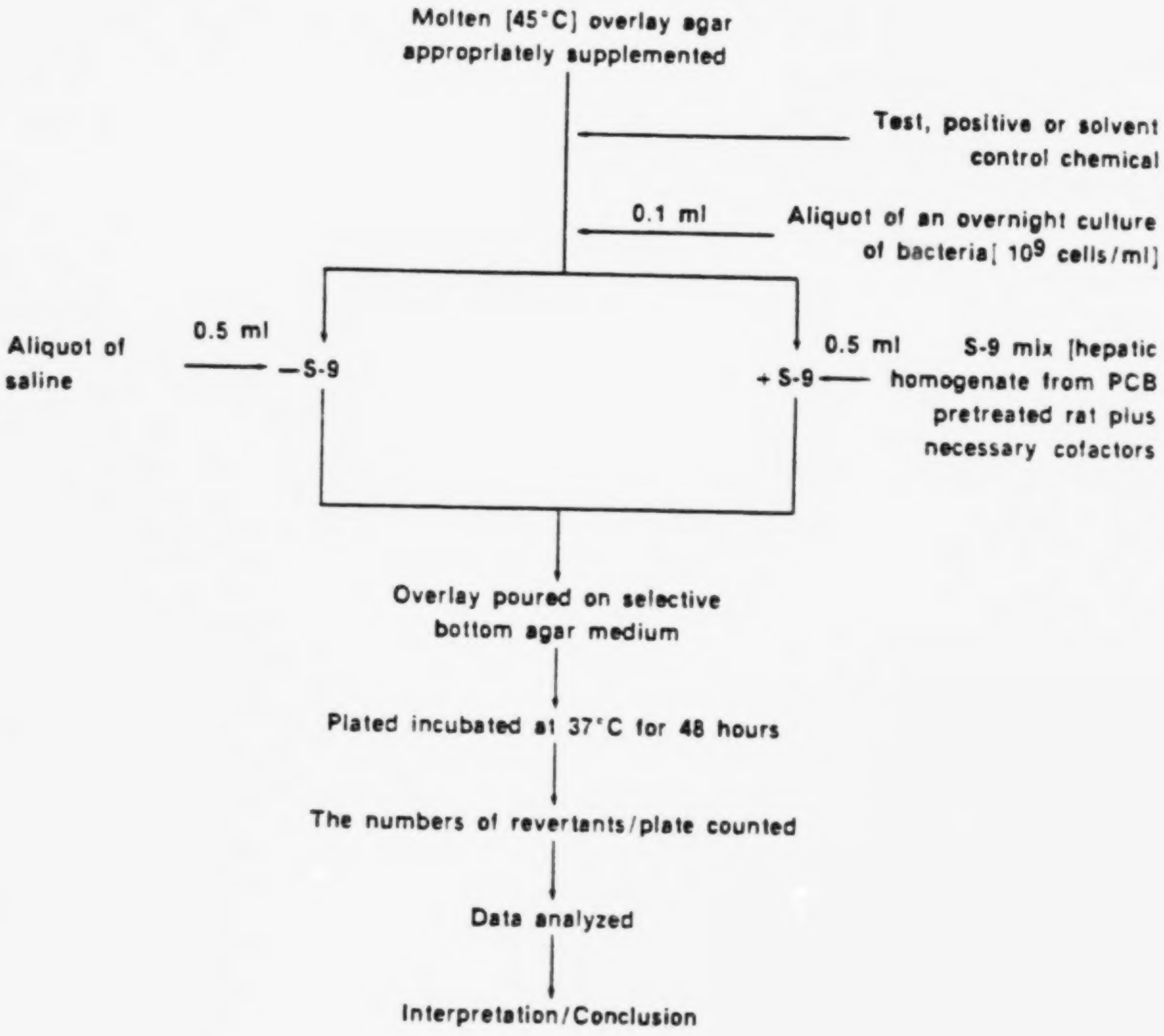
Molten [45°C] overlay agar appropriately supplemented
Test, positive or solvent control chemical
0.1 ml
Aliquot of an overnight culture of bacteria [10^9 cells/ml]
Aliquot of saline
0.5 ml
—S-9
0.5 ml
S-9 mix [hepatic homogenate from PCB pretreated rat plus necessary cofactors
+S-9
Overlay poured on selective bottom agar medium
Plated incubated at 37°C for 48 hours
The numbers of revertants/plate counted
Data analyzed
Interpretation/Conclusion

Figure 1 AMES SALMONELLA/MICROSOME MUTAGENESIS ASSAY

VI. RESULTS

A. Interpretations

The test material, A81-05-030-744 (EA-2 flyash), was dissolved in DMSO at a stock concentration of 100 mg/ml and leached overnight on a shaker at 37°C. Additional dilutions were prepared in DMSO for testing. The maximum test level was 5.0 mg/plate. There was no evidence of toxicity at this level.

Reverse mutation was measured in strains TA-1535, TA-1537, TA-98 and TA-100. The test was conducted in duplicate both with and without rat liver S9 mix for metabolic activation.

There was no mutagenic activity associated with the test material treatment and the sample was considered nonmutagenic and non toxic. The sample was ranked as having nondetectable (ND) mutagenic activity using the IERL-EPA Level 1 evaluation criteria for the Ames assay[1].

Solvent control and positive control values were within acceptable ranges. These results achieved assay acceptance criteria and provided confidence in the assumptions that the recorded data represented typical responses to the test material.

B. Tables

This report is based on the data provided in Table 1.

```
 RESULTS                                            TABLE 1
--------------------------------------

A.    NAME OR CODE DESIGNATION OF THE TEST COMPOUND:  A81-05-030-744 (EA-2 FYLASH)
B.    SOLVENT:  DMSO
C.    TEST INITIATION DATES:  09/24/81
D.    TEST COMPLETION DATE: 09/28/81
E.    S-9 LOT#: S-9 11
NOTE:    CONCENTRATIONS ARE GIVEN IN MILLIGRAMS    PER  PLATE

                                  R E V E R T A N T S   P E R   P L A T E
                                ---------------------------------------------------------------
TEST                 SPECIES TISSUE  TA-1535         TA-1537         TA-98           TA-100
----                 ------- ------ --------------- --------------- --------------- ---------------
                                      1    2    3     1    2    3     1    2    3     1    2    3
NONACTIVATION
-------------

                        ---     ---
SOLVENT CONTROL         ---     ---    12   17          9   12         46   38         132  106
POSITIVE CONTROL**      ---     ---  1076  961        621  628        745  811        1308 1359

TEST COMPOUND
      0.050    MG       ---     ---    10   17         11   11         49   39         162  149
      0.100    MG       ---     ---    10   15          9    8         47   40         153  139
      0.500    MG       ---     ---    11   12         11    5         53   45         130  124
      1.000    MG       ---     ---    12   10         11   14         29   44         151  135
      2.500    MG       ---     ---    11    9         12   16         34   31         140  140
      5.000    MG       ---     ---     9   21         17   13         26   31         141  131

ACTIVATION
----------

SOLVENT CONTROL        RAT     LIVER   17   11         13    8         45   34         101  123
POSITIVE CONTROL***    RAT     LIVER  308  254        339  372       1562 1600        2065 1832

TEST COMPOUND
      0.050    MG      RAT     LIVER   15   13         11   10         54   46         128  163
      0.100    MG      RAT     LIVER    7   12         12   20         46   34         137  140
      0.500    MG      RAT     LIVER   14   11         11   10         62   54         149  111
      1.000    MG      RAT     LIVER   13    9         13   12         49   44         134  127
      2.500    MG      RAT     LIVER    2    8          9    7         37   45         140  136
      5.000    MG      RAT     LIVER    8    7         13   10         47   48         125  142
-----------------------------------------------------------------------------------------------

**                                                         ***
   TA-1535  SODIUM AZIDE                    10 UG/PLATE        TA-1535  2-ANTHRAMINE  2.5 UG/PLATE
   TA-1537  9-AMINOACRIDINE                 50 UG/PLATE        TA-1537  2-ANTHRAMINE  2.5 UG/PLATE
   TA-98    2-NITROFLUORENE                 10 UG/PLATE        TA-98    2-ANTHRAMINE  2.5 UG/PLATE
   TA-100   SODIUM AZIDE                    10 UG/PLATE        TA-100   2-ANTHRAMINE  2.5 UG/PLATE
   SOLVENT  50 UL/PLATE
```

VII. ASSAY ACCEPTANCE AND EVALUATION CRITERIA

Statistical methods are not currently used, and evaluation is based on the criteria included in this protocol.

Plate test data consists of direct revertant colony counts obtained from a set of selective agar plates seeded with populations of mutant cells suspended in a semisolid overlay. Because the test material and the cells are incubated in the overlay for approximately 2 days and a few cell divisions occur during the incubation period, the test is semiquantitative in nature. Although these features of the assay reduce the quantitation of results, they provide certain advantages not contained in a quantitative suspension test:

- The small number of cell divisions permits potential mutagens to act on replication DNA, which is often more sensitive than nonreplicating DNA.
- The combined incubation of the test article and the cells in the overlay permits constant exposure of the indicator cells for approximately 2 days.

A. Surviving Populations

Plate test procedures do not permit exact quantitation of the number of cells surviving chemical treatment. At low concentrations of the test material, the surviving population on the treatment plates is essentially the same as that on the negative control plate. At high concentrations, the surviving population is usually reduced by some fraction. Our protocol will normally employ several doses ranging over two or three log concentrations, the highest of these doses being selected to show slight toxicity as determined by subjective criteria.

B. Dose-Response Phenomena

The demonstration of dose-related increased in mutant counts is an important criterion in establishing metagenicity. A factor that might modify dose-response results for a mutagen would be the selection of doses that are too low (usually mutagenicity and toxicity are related). If the highest dose is far lower than a toxic concentration, no increases may be observed over the dose range selected. Conversely, if the lowest dose employed is highly cytotoxic, the test material may kill any mutants that are induced, and the test material will not appear to be mutagenic.

C. Control Tests

Positive and negative control assays were conducted with each experiment and consisted of direct-acting mutagens for nonactivation assays and mutagens that require metabolic biotransformation in activation assays.

Negative controls consisted of the test material solvent in the overlay agar together with the other essential components. The negative control plate for each strain gave a reference point to which the test data was compared. The positive control assay was conducted to demonstrate that the test systems were functional with known mutagens.

The following normal range of revertants for solvent controls are generally considered acceptable.

TA-1535:	8-30
TA-1537:	4-30
TA-98:	20-75
TA-100:	80-250

D. Evaluation Criteria for Ames Assay

Because the procedures to be used to evaluate the mutagenicity of the test material are semiquantitative, the criteria to be used to determine positive effects are inherently subjective and are based primarily on a historical data base. Most data sets will be evaluated using the following criteria.

1. Strains TA-1535 and TA-1537

If the solvent control value is within the normal range, a test material that produces a positive dose response over three concentrations with the highest increase equal to three times the solvent control value will be considered to be mutagenic.

2. Strains TA-98 and TA-100

If the solvent control value is within the normal range, a test material that produces a positive dose response over three concentrations with the highest increase equal to twice the solvent control value for TA-98 and TA-100 will be considered to be mutagenic.

3. Pattern

Because TA-1535 and TA-100 are both derived from the same parental strain (G-46), to some extent there is a built-in redundancy in the microbial assay. In general, the two strains of a set respond to the same mutagen and such a pattern is sought. Generally, if a strain responds to a mutagen in nonactivation tests, it will do so in activation tests.

4. Reproducibility

If a test material produces a response in a single test that cannot be reproduced in additional runs, the initial positive test data lose significance.

The preceding criteria are not absolute, and other extenuating factors may enter into a final evaluation decision. However, these criteria will be applied to the majority of situations and are presented to aid those individuals not familar with this procedure. As the data base is increased, the criteria for evaluation can be more firmly established.

E. Relation Between Mutagenicity and Carcinogenicity

It must be emphasized that the Ames *Salmonella*/Microsome Plate Assay is not a definitive test for chemical carcinogens. It is recognized, however, that correlative and functional relations have been demonstrated between these two endpoints. The results of comparative tests on 300 chemicals by McCann *et al.*[4] show an extremely good correlation between results of microbial mutagenesis tests and *in vivo* rodent carcinogenesis assays.

All evaluations and interpretation of the data to be presented in the final report will be based only on the demonstration, or lack, of mutagenic activity.

F. Criteria for Ranking Samples in the Ames Assay

The goal of EPA Level 1 Ames testing is to rank source streams by relative degree of genetic toxicity (mutagenicity). Samples are first identified as mutagenic or nonmutagenic by the criteria in Section D above and then ranked using the mutagenicity categories presented in the table below. The lowest concentration giving a positive response in any strain, with or without metabolic activation, is identified as the minimum effective concentration (MEC) for that sample. The mutagenicity of the sample is evaluated as high (H), moderate (M), low (L), or nondetectable (ND) according to the evaluation criteria developed in the Level 1 manual[1] and summarized below. Samples with no detectable activity at the maximum applicable dose (MAD) are ranked nondetectable (ND).

Ames Assay Mutagenicity Ranking Criteria[1]

Mutagenic Activity	Solids (MEC in µg/plate)	Liquids[a] (MEC in µl/plate)
High (H)	<50	<2
Moderate (M)	50-500	2-20
Low (L)	500-5000	20-200
Not Detectable (ND)	>5000	>200

[a] Concentration of organic extracts is based upon organic content (µg organics per plate) and not volume (µl extract per plate) of sample tested.

VIII. REFERENCES

1. Brusick, D.J., et al.: IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests. EPA Contract No. 68-02-2681, Technical Directive No. 501, Litton Bionetics, Inc., Kensington, MD, September 1980, 177 pp. In press.

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3. Brusick, D.J. and Young, R.R.: Level 1 Bioassay Sensitivity. EPA-600/7-81-135, Litton Bionetics, Inc., Kensington, MD, August 1981, 52 pp.

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GENETICS ASSAY NO.: 5887
LBI SAFETY NO.: 7171

CYTOTOXIC EVALUATION OF
A81-05-030-744
(EA-2 FLYASH)
IN THE RABBIT
ALVEOLAR MACROPHAGE (RAM)
CYTOTOXICITY ASSAY

FINAL REPORT

SUBMITTED TO:

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SUBMITTED BY:

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LBI PROJECT NO.: 22064

REPORT DATE: NOVEMBER 1981

5-372

Litton BIONETICS

PREFACE

This assay conforms to the standard EPA Level 1 procedure for the rabbit alveolar macrophage (RAM) cytotoxicity assay as described in "IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests" (1). The data were evaluated and formatted as recommended in "Level 1 Biological Testing Assessment and Data Formatting" (2).

The RAM cytotoxicity assay has been shown to be a sensitive method for detecting cytotoxic activity for a variety of chemicals representing various chemical classes (3). This assay is one of several recommended by EPA to identify, categorize and rank the pollutant potential of influent and effluent streams from industrial and energy-producing processes. This assay has been well validated with a wide range of positive and negative control chemicals and complex environmental samples.

All procedures and documents pertaining to the receipt, storage, preparation, testing and evaluation of the test material shall conform to Litton Bionetics, Inc. standard operating procedures and the Good Laboratory Practices Regulations of 1979. Deviations from standard procedure shall be fully documented and noted in the report.

All test and control results in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Molecular Toxicology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20895. Copies of raw data will be supplied to the sponsor upon request.

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I. ASSAY SUMMARY

A. SPONSOR: Acurex Corporation

B. MATERIAL (TEST COMPOUND): GENETICS ASSAY NUMBER: 5887

1. Identification: A81-05-030-744 (EA-2 Flyash)
2. Date Received: August 26, 1981
3. Physical Description: Fine, gray and white particles with shreds of black material.

C. TYPE OF ASSAY: Rabbit Alveolar Macrophage (RAM) Cytotoxicity Assay

D. ASSAY DESIGN NUMBER: 443

E. STUDY DATES:

1. Initiation: September 23, 1981
2. Completion: October 14, 1981

F. SUPERVISORY PERSONNEL:

1. Study Director: Brian Myhr, Ph.D.
2. Laboratory Supervisor: Robert Young, M.S.

G. EVALUATION:

The test material was tested as supplied and after puliverization to a very fine powder. Both forms of the material caused only slight toxicity at concentrations above 500 μg/ml. The most sensitive parameters, ATP content and viability index, indicated EC_{50} values above the MAD level of 1000 μg/ml. Therefore, the results were evaluated as showing nondetectable (ND) toxicity for this test material, according to the IERL-EPA Level 1 toxicity categories in the RAM Cytotoxicity Assay.

Submitted by:

Study Director

Brian Myhr 11/24/81

Brian Myhr, Ph.D. Date
Associate Director,
Department of Molecular
Toxicology

11/24/81

David J. Brusick, Ph.D. Date
Director,
Department of Molecular
Toxicology

II. OBJECTIVE

The objective of this study was to determine and rank the cytotoxicity of A81-05-030-744 (EA-2 Flyash) to cultured rabbit alveolar macrophage (RAM) cells. The measure of cytotoxicity was the reduction in cell viability and adenosine triphosphate (ATP) content of the cultures after a 20 hour exposure to the test material. At the conclusion of the exposure period, the number of viable cells and total ATP content in the treated cultures were compared to the corresponding values in unexposed control cultures. The concentration of test material that reduced each experimental parameter by 50% was estimated graphically and referred to as the EC50 value. Standard EPA Level 1 toxicity evaluation criteria for the RAM cytotoxicity assay were used to rank the toxicity potential of the test material based upon the most sensitive parameter.

III. TEST MATERIAL

A. Description

The test material was received as a gray and white particulate containing thin shreds of black material. The amount of sample supplied was 15 grams. No information on the sampling parameters was provided.

B. Handling and Preparation

The test material was received on August 26, 1981, and was assigned LBI assay number 5887 and LBI safety number 7171. The sample was stored at +4°C in the dark.

Approximately 34 mg of the test material was tested as supplied. Then on October 1, 1981, the remaining sample was ground in a mortar and pestle to fine black powder. Approximately 2.5 grams of the ground sample was further pulverized on October 8, 1981, to a very fine, black powder of which 30 mg was used in the second trial of the assay. For both trials, the test material was suspended in serum-free EMEM culture medium at a concentration of 2000 µg/ml and incubated at 37°C on a roller drum for 8 hours. The original material settled quickly on standing, but the suspension formed from the pulverized powder remained well-dispersed for dilutions. No pH changes were observed. The suspensions were serially diluted with EMEM (serum-free) and applied to the cultures at a maximum concentration of 1000 µg/ml in the presence of 10% serum.

IV. MATERIALS

A. Indicator Cells

The two trials employed short-term primary cultures of alveolar macrophage cells obtained by lung lavages of male New Zealand white rabbits (2.0-2.5 kg). The rabbits were maintained on Purina Lab Rabbit Chow 5321 and water *ad libitum* and were examined for the absence of respiratory illnesses prior to use.

B. Media

The cells were maintained and treated in Eagle's Minimum Essential Medium (EMEM) with Earle's salts and supplemented with 10% fetal bovine serum (heat-inactivated), 100 units/ml penicillin, 100 µg/ml streptomycin, 17.6 µg/ml kanamycin, and 0.4 µg/ml amphotericin B.

C. Negative Controls

The negative control for the first trial consisted of six untreated cultures carried through the same experimental time period as the treated cells. Six cultures were used because a large number of cells was obtained by pooling the yield from two rabbits in order to run two concurrent assays. Only one animal was used for the second trial, and the usual three untreated cultures were prepared. The average viability and ATP content of the negative controls provided the reference points for determining the effects of different concentrations of the test material on the assay parameters.

V. EXPERIMENTAL DESIGN

A. Procurement of Cells

The rabbits were sacrificed by injection of Nembutal® (60 mg/ml) into the marginal ear vein, and sterile operating techniques were used to perform a tracheostomy. Prewarmed normal saline (30 ml) was then introduced into the lungs via a catheter and allowed to stand for 15 minutes. This lavage fluid was removed and placed into a 50-ml sterile centrifuge tube on ice. Nine additional lavages were similarly performed and collected, except the saline was removed shortly after its introduction into the lungs. Any lavage fluid containing blood or mucous was discarded. The lavages were centrifuged at 365 x g for 15 minutes and the cells resuspended in cold 0.85% saline. After two washes in saline by centrifugation, the cell pellets were resuspended in cold EMEM containing 20% serum and then combined. A cell count was obtained by hemocytometer and the suspension diluted to between 5×10^5 and 10^6 cells/ml. Viability was determined by trypan blue staining and the cells were not used if less than 95% viable. Also, a differential cell count from Wright-stained smears was performed to verify that the macrophage content was above 90%.

B. Sample Forms

The usual sample form for application to the cells is a suspension of particulate material. Solid samples are ground to fine particles and a weighed portion is suspended in a known volume of EMEM (0% FBS) for about eight hours to help leach any water-soluble material. Finely-divided test material may be suspended directly in culture medium without further grinding. Aqueous liquids, suspensions, or slurries containing less than 0.5% organic solvent are added by volume to culture medium.

Samples supplied as solutions in organic solvents are usually solvent-exchanged into DMSO before testing. Original sample volumes may first be reduced a maximum of 10-fold in a Kuderna-Danish concentrator, and the concentrative factor is used to convert assayed volumes into equivalent original sample volumes in the absence of information about solute concentration. An aliquot of the reduced volume is exchanged into DMSO by repeated, partial evaporation under a stream of nitrogen in a warm water bath (50°C); the evaporated volumes are replaced with equal volumes of DMSO.

Samples adsorbed on XAD-2 resin are extracted with methylene chloride or acetone in a Soxhlet apparatus for 24 hours. The extract is then concentrated and solvent-exchanged into DMSO. Alternatively, acetone extracts can be assayed directly at concentrations up to 2% by volume in the culture medium.

Samples impregnated on fiber glass or teflon filters are repeatedly sonicated in cyclohexane to remove particulates. The resulting cyclohexane particulate suspension is then evaporated to dryness and the particulates resuspended in EMEM culture medium at the desired concentration.

Sponsor-specified handling of sample materials will be followed if the above procedures are not applicable or a specific procedure is desired.

C. Dose Selection

Unless the approximate toxicity is already known or the sample size is limiting, the following usual dose ranges are tested for different sample forms. Dry, particulate material is tested at six dose levels from 1000 µg/ml to 3 µg/ml. Aqueous samples, suspensions, or slurries are tested from 600 µl to 3 µl/ml in six dose steps. Samples that are solvent-exchanged into DMSO are tested from 20 µl/ml (2% DMSO in growth medium) to 0.2 µl/ml, also in six dose steps. A second dose study is performed with an adjusted dose range if the EC50 was not located properly in the initial test. However, EC50 values greater than 1000 µl/ml for particulate material, 600 µl/ml for aqueous samples, or 20 µl/ml for organic solutions will not be determined.

This test material, A81-05-030-744 (EA-2 flyash), was tested as supplied at 6 dose levels in the first trial, starting at the maximum applicable dose (MAD) of 1000 µg/ml and including 600, 300, 100, 60 and 30 µg/ml. The second trial was performed with only three doses of the finely ground test material: 1000, 600 and 300 µg/ml.

D. Treatment

A series of 25 cm^2 culture flasks were prepared, each containing 2.0 ml of serum-free medium at 37°C and the test material at twice the desired final concentration. Three flasks were prepared for each test concentration. Aliquots of cell suspension (2 ml) were then added; each flask, therefore, contained from 1 to 2 x 10^6 viable cells in a 4-ml volume of media containing 10% serum. The flasks were placed on a rocker platform in a 37°C incubator with a humidified atmosphere containing 5% CO_2. After sitting for about 30 minutes, the flasks were slowly rocked for the remainder of a 20-hour exposure period.

If the test substance causes a color change in the growth medium, the pH is determined in additional treated flasks. After the exposure period, the pH of the medium in the experimental flasks is again recorded.

E. Cell Viability Assay

At the end of the treatment period, the medium containing unattached cells was decanted into a centrifuge tube on ice. The attached cells were rinsed with 1 ml of 0.1% trypsin/0.01% versene and then incubated with 2 ml of the trypsin/versene solution for about 5 minutes at 37°C. The trypsinates and decanted media were combined for each culture to yield a 7-ml cell suspension for subsequent analysis.

A 0.5 ml or 1.0 ml aliquot of the cell suspension was removed for cell count and viability determination. The aliquot was combined with 1.0 ml of 0.4% trypan blue and counted by hemocytometer about 5 to 15 minutes later. The total number of cells counted per culture was the sum of the numbers found in five squares for each chamber of the hemocytometer (1 μl total volume). The numbers of live (colorless) and dead (blue) cells were recorded.

F. ATP Assay

ATP was immediately analyzed by extraction of a 0.1-ml sample of cell suspension with 0.9 ml of 90% DMSO. After 2 minutes at room temperature 5.0 ml cold MOPS buffer (0.01 M morpholinopropane sulfonic acid) at pH 7.4 was added and the extract mixed well and placed on ice. Aliquots of 10 μl were injected into a cuvette containing a luciferin-luciferase reaction mixture in a DuPont Model 760 Luminescence Biometer. The Biometer was calibrated daily with standard ATP solutions to provide a direct read-out of the ATP content. Each test sample was assayed at least twice to obtain repeatable readings.

VI. ASSAY ACCEPTANCE CRITERIA

The assay will be considered acceptable for evaluation of the test results if the following criteria are met:

1. The macrophage population is 90% or greater of the total nucleated cells collected by lung lavage.
2. The percent viability of the macrophages used to initiate the assay is 95% or greater.
3. The survival of viable macrophages in the negative control cultures over the 20 hour treatment priod is 70% or greater.
4. A sufficient number of data points (for five test concentrations or less) are available to clearly locate the EC50 of the most sensitive test parameter within a toxicity region as defined under Assay Evaluation Criteria.
5. The data points critical to the location of the EC50 for the most sensitive parameter are the averages of at least two treated cultures.
6. If all the test parameters yield EC50 values greater than 1000 µg/ml, 600 µl/ml for aqueous solutions, or 20 µl/ml for organic solutions, the plotted curves for ATP content and viability index parameters do not exceed 120% of the negative control.

VII. RESULTS

A. Interpretation

Two trials were performed to test the effect of puliverization on the toxicity of the test material to the RAM cells. The original test material consisted of a fine powder and long, thin shreds of black material, and the test results for this material are presented in Table 1 and Figures 1 and 2. When the test material was puliverized to a very fine powder, the results shown in Table 2 and Figures 3 and 4 were obtained. Absolute and relative assay parameters are provided in the tables, whereas the relative values are plotted in the figures to determine EC_{50} positions.

In both assays, the test parameters remained above 70% of the negative control values for all applied dosed up to the MAD level of 1000 µg/ml. Some toxicity was indicated in the 100-1000 µg/ml concentration range by the viability index and the ATP content, but the decreased in these parameters were insufficient to ascribe toxic properties to the test material. Pulverization of the test material appeared to slightly reduce the toxicity, if it did anything, perhaps by eliminating the long thin strands of material that could pierce the cells after being englufed. Since the most sensitive assay parameters (ATP content and viability index) indicated EC_{50} values above 1000 µg/ml, the test material was evaluated as having nondetectable (ND) toxicity, according to the toxicity categories defined for the IERL-EPA Level 1 RAM assay[1].

The macrophages collected for both assays had normal morphology and appeared to be in a healthy state. The initial viability was approximately 99% and the survival of viable cells in the negative controls for both trials was at least 96 percent. The average cellular ATP content of the negative control (ATP/10^6 total cells) of the negative controls was within the historical range for acceptable cultures in both assays. These results achieved the assay acceptance criteria and provided confidence in the assumption that the collected data represented typical responses to the test material.

B. Tables and Figures

This report is based on the data provided in Tables 1 and 2 and Figures 1 to 4.

TABLE 1
RABBIT ALVEOLAR MACROPHAGE (RAM) CYTOTOXICITY ASSAY DATA

LBI Assay No.: 5887 (Trial I, Unground sample)

Test Material Identity: A81-05-030-744 (EA-2 Flyash)

Test Date: September 23, 1981

Vehicle: EMEM

Initial Cell Viability: 98.8%

Viable Macrophage Seeded/Flask: 2.0×10^6 cells/flask

Macrophage Population Percentage: >90.0%

Survival of Negative Control Macrophage Over Treatment Time: 99.1%

		Average Values per Culture Flask					Expressed as Percent of Negative Control			
Sample	Concentration[a] µg/ml	Viable Cells 10^6 Units	Total Cells 10^6 Units	ATP 10^8 fg[b]	ATP Per 10^6 Cells 10^8 fg	Viability %	Viability	Viability Index	ATP	ATP Per 10^6 Cells
NC[c]	---	2.14	2.16	66.4	30.7	99.1	100.0	100.0	100.0	100.0
TEST	30	1.91	1.92	67.6	35.2	99.5	100.4	89.3	101.8	114.7
TEST	60	1.96	2.00	65.5	32.8	98.0	98.9	91.6	98.6	106.8
TEST	100	1.83	1.86	65.9	35.4	98.4	99.3	85.5	99.2	115.3
TEST	300	1.91	2.04	64.7	31.7	93.6	94.5	89.3	97.4	103.3
TEST	600	1.53	1.63	56.7	34.8	93.9	94.8	71.5	85.4	113.4
TEST	1000	1.62	1.89	47.0	24.9	85.7	86.5	75.7	70.8	81.1
					[d]EC50 VALUES: µg/ml:		>1000	>1000	>1000	>1000

Toxicity Classification: Nondetectable

[a] pH change in culture medium: None observed

[b] fg = Femtogram (10^{-15} gram).

[c] NC = Negative Control, EMEM culture medium.

[d] Determined from data plots in Figures 1 and 2.

TABLE 2

RABBIT ALVEOLAR MACROPHAGE (RAM) CYTOTOXICITY ASSAY DATA

LBI Assay No.: 5887 (Trial II Ground sample)

Test Material Identity: A81-05-030-744 (EA-2 Flyash)

Test Date: October 13, 1981

Vehicle: EMEM

Initial Cell Viability: 99.3%

Viable Macrophage Seeded/Flask: 1 x 10^6 cells/flask

Macrophage Population Percentage: >90.0%

Survival of Negative Control
Macrophage Over Treatment Time: 96.0%

Sample	Concentration[a] µg/ml	Average Values per Culture Flask Viable Cells 10^6 Units	Average Values per Culture Flask Total Cells 10^6 Units	Average Values per Culture Flask ATP 10^8fg[b]	ATP Per 10^6 Cells 10^8 fg	Viability %	Expressed as Percent of Negative Control Viability	Expressed as Percent of Negative Control Viability Index	Expressed as Percent of Negative Control ATP	Expressed as Percent of Negative Control ATP Per 10^6 Cells
NC[c]	---	0.97	1.01	25.4	25.1	96.0	100.0	100.0	100.0	100.0
TEST	300	0.90	0.95	22.4	23.6	94.7	98.6	92.8	88.2	94.0
TEST	600	0.83	0.86	22.7	26.4	96.5	100.5	85.6	89.4	105.2
TEST	1000	0.75	0.80	21.1	26.4	93.8	97.7	77.3	83.1	105.2

[d] EC50 VALUES:
µg/ml: >1000 >1000 >1000 >1000

Toxicity
Classification: Nondetectable

---

[a] pH change in culture medium: None observed

[b] fg = Femtogram (10^{-15} gram).

[c] NC = Negative Control, EMEM culture medium.

[d] Determined from data plots in Figures 3 and 4.

FIGURE 1

EC50 DETERMINATION FOR

PERCENT VIABILITY (O) AND VIABILITY INDEX (●)

A81-05-030-744

(EA-2 FLYASH)

TRIAL 1

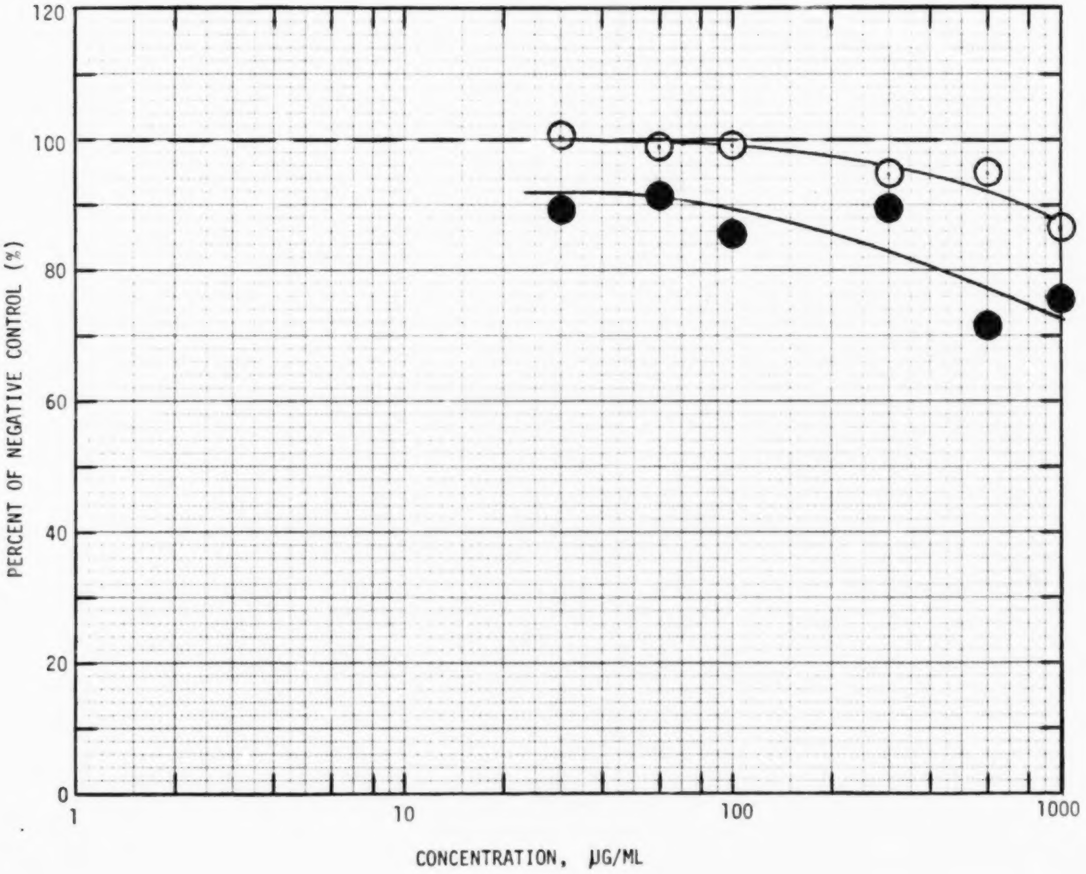


FIGURE 2

EC50 DETERMINATION FOR

ATP/FLASK (O) AND ATP/10^6 CELLS (●)

A81-05-030-744

(EA-2 FLYASH)

TRIAL 1

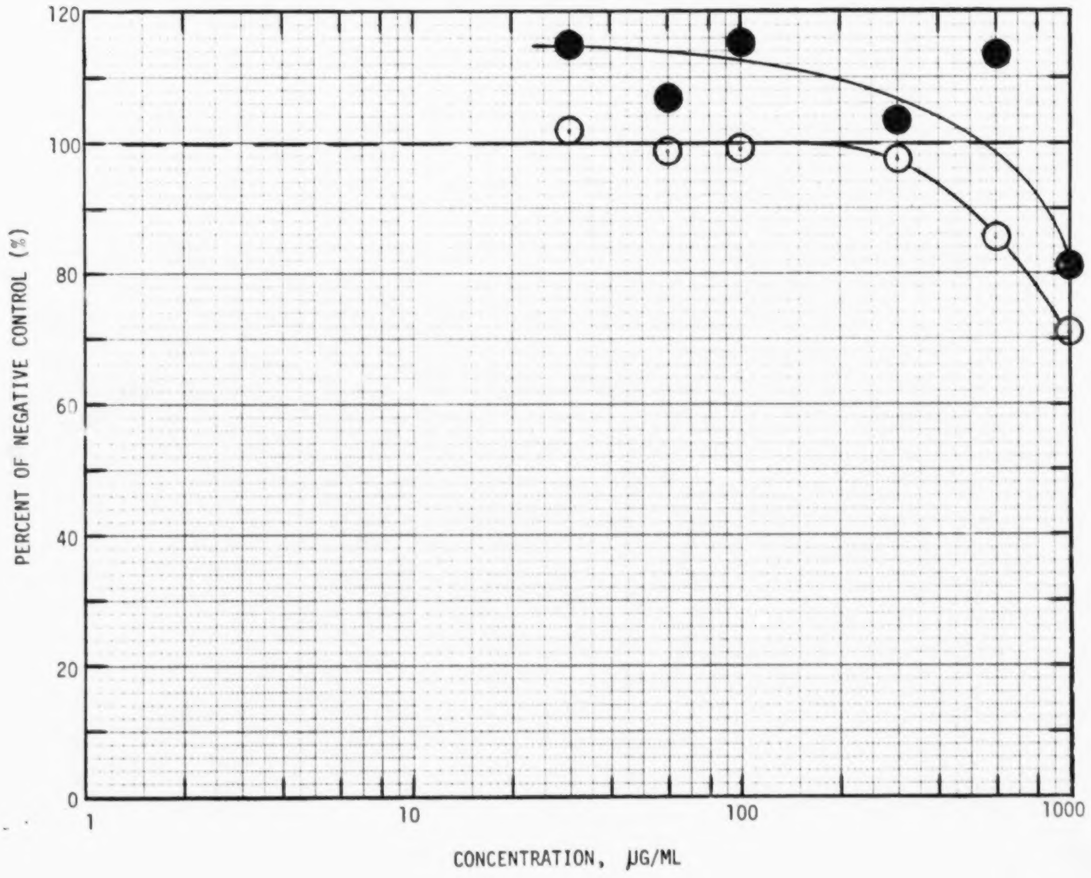


FIGURE 3

EC50 DETERMINATION FOR

PERCENT VIABILITY (O) AND VIABILITY INDEX (●)

A81-05-030-744
(EA-2 FLYASH)
TRIAL 2

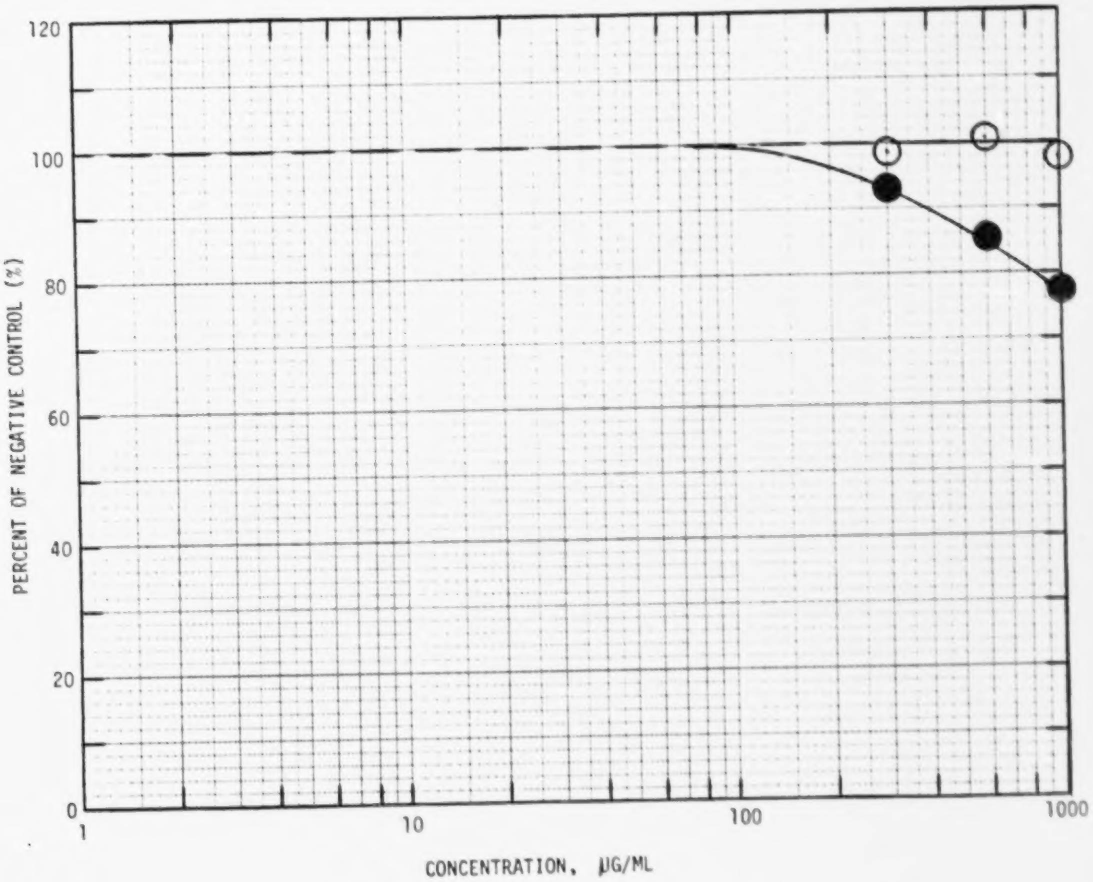


FIGURE 4

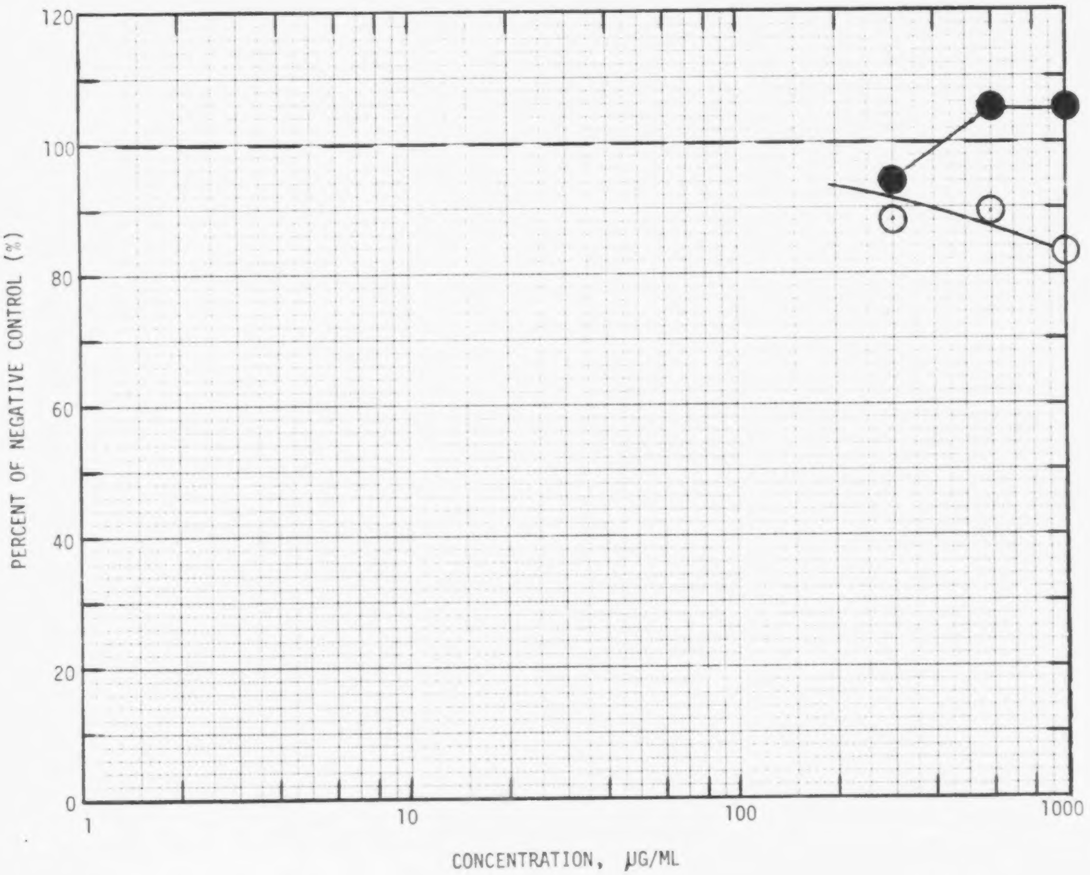
EC50 DETERMINATION FOR

ATP/FLASK (O) AND ATP/10^6 CELLS (●)

A81-05-030-744

(EA-2 FLYASH)

TRIAL 2



VIII. ASSAY EVALUATION CRITERIA

The EC50 value represents the concentration of test material that reduces the most sensitive parameter of the RAM assay to 50% of the vehicle or negative control value. EC50 values are determined graphically by fitting a curve by eye through relative toxicity data plotted as a function of the logarithm of the applied concentration. Each data point normally represents the average of three culture dishes. Statistical analysis is unnecessary in most cases for evaluation.

The toxicity of the test material is evaluated as high, moderate, low, or nondetectable according to the range of EC50 values defined in the following table.

Toxicity[a]	Solids (EC_{50} in μg/ml)	Aqueous Liquids (EC_{50} in μl/ml)	Nonaqueous Liquids[b] (EC_{50} in μl/ml)
High	<10	<6	<0.2
Moderate	10 to 100	6 to 60	0.2-2
Low	100 to 1000	60 to 600	2-20
Not Detectable	>1000	>600	>20

[a] Evaluation criteria formulated by Litton Bionetics, Inc. for IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests[1].

[b] Criteria for nonaqueous liquids are tentative and under evaluation. If the organic or solid content is known, the solid evaluation criteria are applied.

Another evaluation scheme is proposed for extracts obtained from SASS train gas volumes. The proportion of the total gas volume corresponding to the volume of extract used in the bioassay is calculated and expressed as L/ml of culture medium (or DSCF/ml of culture medium). A criterion of 1000 L/ml is set as the limit for nondetectable toxicity. This gas volume corresponds to the average volume breathed by humans over a 2-hour period. The subsequent toxicity ranges are defined by 10-fold dilution steps to conform to standard procedure. The toxicity ranges are defined in the following table for liter and dry standard cubic feet units:

Toxicity	EC_{50} In Liters/ml (L/ml)	EC_{50} In Dry Standard Cubic Feet/ml (DSCF/ml)
High	<10	<0.35 DSCF
Moderate	10-100	0.35-3.5
Low	100-1000	3.5-35
Nondetectable	>1000	>35

IX. REFERENCES

1. Brusick, D.J., et al.: IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests. EPA Contract No. 68-02-2681, Technical Directive No. 501, Litton Bionetics, Inc., Kensington, MD, September 1980, 177 pp. In press.

2. Brusick, D.J.: Level 1 Bioassay Assessment and Data Formatting. EPA-600/7-80-079, Litton Bionetics, Inc., Kensington, MD, April 1980, 100 pp.

3. Brusick, D.J. and Young, R.R.: Level 1 Bioassay Sensitivity. EPA-600/7-81-135, Litton Bionetics, Inc., Kensington, MD, August 1981, pp. 52.

GENETICS ASSAY NO.: 5887
LBI SAFETY NO.: 7171

TOXIC EVALUATION OF A81-05-030-744 (EA-2 FLYASH) IN THE EPA LEVEL 1 ACUTE IN VIVO RODENT TOXICITY ASSAY

FINAL REPORT

SUBMITTED TO:

ACUREX CORPORATION
485 CLYDE AVENUE
MOUNTAIN VIEW, CALIFORNIA 94042

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MD 20795

LBI PROJECT NO.: 22064

REPORT DATE: NOVEMBER 1981

Litton BIONETICS

PREFACE

This assay conforms to the standard EPA Level 1 procedure for the acute _in vivo_ toxicity test in rodents as described in "IERL-RTP Procedures _Manual_: Level 1 Environmental Assessment Biological Tests"[1]. The data were evaluated and formatted as recommended in "Level 1 Biological Testing Assessment and Data Formatting"[2]. The organisms used in this assay were male and female weanling mice as recommended by the Level 1 Manual.[1]

The advantages of _in vivo_ toxicity assays are embodied mainly in the fact that the toxicological assessment is performed in whole animals. There is a significant background of test data on a wide range of toxicants for the rodent systems, thus supplying needed information for the reliable interpretation of results with complex effluents[3]. The main disadvantage of an acute rodent toxicity study is a possibly unsatisfactory prediction of toxicity induced by long-term/ low-level exposures. An additional consideration is the need for multi-gram quantities of test material which may prohibit testing where small amounts of sample are available, such as from source streams containing gaseous and particulate material.

Since the major objective of the Level 1 biological testing procedures is to identify toxicological problems at minimal cost, a two-step approach was developed for the initial acute _in vivo_ toxicological evaluation of unknown compounds. The first step is based on the quantal (all-or-none) response of dosing animals only at the maximum applicable dose. If no animals die in the quantal test, further _in vivo_ testing is not initiated and the sample toxicity is categorized as _not detectable_. If any animals die in the quantal screening, a multiple dose quantitative test is initiated to determine the dose that kills 50 percent of the animals (LD_{50}). The toxicity potential of the test material is then ranked using standard EPA Level 1 toxicity evaluation criteria for the acute _in vivo_ rodent toxicity assay[1].

All procedures and documents pertaining to the receipt, storage, preparation, testing and evaluation of the test material shall conform to Litton Bionetics, Inc. standard operating procedures and the Good Laboratory Practices Regulations of 1979. Deviations from standard procedure shall be fully documented and noted in the report.

All test and control results in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Molecular Toxicology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20795. Copies of raw data will be supplied to the sponsor upon request.

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I. ASSAY SUMMARY

A. SPONSOR: Acurex Corporation

B. MATERIAL (TEST COMPOUND): GENETICS ASSAY NO.: 5887

1. Identification: A81-05-030-744 (EA-2 Flyash)
2. Date Received: August 26, 1981
3. Physical Description: Gray and white particles with much larger, long and thin black chunks.

C. TYPE OF ASSAY: EPA Level 1 Rodent Quantal Toxicity Assay

D. STUDY DATES:

A. Initiation: October 5, 1981

B. Completion: October 23, 1981

E. SUPERVISORY PERSONNEL:

A. Study Director: David J. Brusick, Ph.D.

B. Senior Technician: Joan McGowan

F. EVALUATION:

The test substance, A81-05-030-744 (EA-2 Flyash), was not lethal or toxic for weanling mice following an oral gavage dose of 5 gm/kg body weight. Although one female animal was found dead, the death did not appear compound-related because of the absence of toxic signs. Otherwise there were no unusual findings upon necropsy that would suggest test substance related toxicity. The LD_{50} of the test material was found to be higher than the maximum applicable dose (MAD) of 5 gm/kg. The test sample response was evaluated as being in the nondetectable range as defined for the IERL-EPA Level 1 Rodent Quantal Toxicity Assay[1].

Submitted by:

David J. Brusick, Ph.D.
Director
Department of Molecular
Toxicology

11/22/81
Date

II. OBJECTIVES

The objective of this assay was to evaluate the acute toxicity of sample A81-05-030-744 (EA-2 flyash) when administered by oral gavage to male and female weanling mice. Test strategy involved initial testing of the sample at the maximum applicable dose in the quantal assay. If lethality was observed in the quantal assay, additional testing would be initiated at lower doses to identify the LD_{50}.

The assay consisted of recording any lethality and toxic signs that occurred initially and over a 14-day period following a single treatment. Additional information was collected from necropsy observations on animals that died during the course of the experiment or were killed at the end of the 14-day observation period.

III. TEST MATERIAL

A. Description

The test material, A81-05-030-744 (EA-2 flyash), was received as gray and white particles with larger (long and thin) black chunks. The amount of sample supplied was 15.0040 grams. No information on the sampling parameter was provided.

B. Handling and Preparation

The test material was received at LBI on August 26, 1981. The sample was assigned LBI safety number 7171 and LBI assay number 5887. The sample was stored at +4°C in the dark.

On October 1, 1981, the test material was ground in a mortar and pestle to a fine, black powder. The primary dosing suspension was prepared 24 hours in advance to permit water soluble materials in the flyash to leach into the water at room temperature. A total of 1628.31 mg of test material was suspended in 17.43 ml of sterile distilled water giving a stock concentration of 93.6 mg/ml. This suspension would not pass freely through a 24G gavage needle so it was discarded. On October 8, 1981, approximately 2.5 gm of the previously ground sample was puliverized a second time in a mortar and pestle. The suspension, prepared 24 hours in advance of dosing, passed through the gavage needle without difficulty. A total of 1411.04 mq of test material was suspended in 10.1 ml of sterile water giving a stock concentration of 140 mg/ml.

IV. MATERIALS

A. Test Organisms

The test organisms for this study were weanling Charles River CD-1 mice. Weanlings were used because they are likely to be more sensitive to toxic effects of test materials than adult mice. In addition, significantly less test material is required for dosing.

Eight nursing female Charles River CD-1 mice with ten pups each (5 male and 5 female) were obtained from Charles River Breeding Laboratories, Inc., Wilmington, MA on September 30, 1981. The birth date of the pups was September 13, 1981. The animals were quarantined for 5 days upon receipt. The litters were individually housed on Ab-sorb-dri bedding in polycarbonate cages and were cared for according to Litton Bionetics, Inc., Department of Molecular Toxicology and LAMS Standard Operating Procedures. Purina certified laboratory chow and water (pH 2.5) were provided *ad libitum*. The pups were maintained with mothers until weaned. The animals were identified by eartags and cage cards and were released for study on October 9, 1981.

V. EXPERIMENTAL DESIGN

A. Quantal Test

Ten male and ten female weanling CD-1 mice were used in the initial quantal screening test. The pups appeared to be in good health with no physical or behavioral problems noted. Pups that were selected were of similar size. The pups were 26 days old at the time of dosing.

Prior to dosing, each animal was individually weighed and the mean weight calculated for each sex. The volume of test material to be administered was based on the mean weight if all animals were within plus or minus 15 percent of the average for the sex. If any animals were outside that range, they were then excluded from the average, a new mean calculated for the remaining animals and individual dosing volumes calculated for each outlying animals.

The test material was administered by gavage to the pups at the rate of 5 gm/kg. The average weight of the males was 11.5 grams and that of the females was 12.0 grams. The weight of one female, animal number 9058, exceeded ±15 percent of the average of the females. This animal was excluded, and the new average of 11.8 grams calculated for the females. The test material, suspended at a concentration of 140 mg/ml, was applied to the animals in two equal doses that totaled 0.41 ml for the males, 0.51 for the females, except animal number 9058 that received 0.42 ml.

Immediately following administration of the test substance and during the first day, observations of the frequency and severity of all toxic signs or pharmacological effects (Table 1) were recorded. Particular attention was paid to time of onset and disappearance of signs. Observations were made and recorded on all animals through a 14-day period. At termination of the observation period, all surviving animals were weighed, killed, and then gross necropsies performed. Necropsies were also performed on all animals that died during the course of this study.

B. Quantitative Test

Since no animals died during the preliminary quantal screening test, the quantitative test to determine the LD_{50} was unneccessary.

TABLE 1. DEFINITION OF PHARMACOLOGICAL TOXIC SIGNS

Organ System	Observation and Examination	Common Signs of Toxicity
CNS and somatomotor	Behavior	Change in attitude to observer, unusual vocalization, restlessness, sedation
	Movements	Twitch, tremor, ataxia, catatonia, paralysis, convulsion, forced movements
	Reactivity to various stimuli	Irritability, passivity, anaesthesis, hyperaesthesis
	Cerebral and spinal reflexes	Sluggishness, absence
	Muscle tone	Rigidity, flaccidity
Autonomic nervous system	Pupil size	Myosis, mydriasis
	Secretion	Salivation, lacrimation
Respiratory	Nostrils	Discharge
	Character and rate of breathing	Bradypnoea, dyspnoea, Cheyne-Stokes breathing, Kussmaul breathing
Cardiovascular	Palpation of cardiac region	Thrill, bradycardia, arrhythmia, stronger or weaker beat
Gastrointestinal	Events	Diarrhea, constipation,
	Abdominal shape	Flatulence, contraction
	Feces consistency and color	Unformed, black or clay colored
	Vulva, mammary glands	Swelling
	Penis	Prolapse
	Perianal region	Soiled
Skin and fur	Color, turgor, integrity	Reddening, flaccid skinfold, eruptions, piloerection
Mucous membranes	Conjunctiva, mouth	Discharge, congestion, hemorrhage cyanosis, jaundice
Eye	Eyeball	Exophthalmus, nystagmus
	Transparency	Opacities
Others	Rectal or paw skin	Subnormal, increased temperature
	General Condition	Abnormal posture, emaciation

VI. RESULTS

A. Interpretation

The test material, A81-05-030-744 (EA-2 flyash), was tested and evaluated in the EPA Level 1 Acute *In Vivo* Rodent Toxicity Assay. The first phase of testing was the quantal toxicity test in which 10 male and 10 female weanling CD-1 mice were exposed to an oral dose of the test material. This dose was at the maximum applicable dose (MAD) of 5 gm/kg as recommended by the EPA Level 1 procedures manual[1].

Nineteen animals survived the exposure with no evidence of any significant compound-related behavioral or toxic signs (see Table 1 for definitions). The animals seemed uncomfortable after dosing (slow moving, wiping mouth and eyes half-shut) but animals appeared normal after 2 hours. There was a small amount of test material on the muzzle of some animals after dosing. One animal, female number 9053, was found dead on day 3 of the study. The animal had been dead a number of hours; rigor mortis had set in and the intestines were filled with gas. Necropsy of animal 9053 indicated necrosis of the liver but no other gross lesions. The death of this animal did not appear directly attributable to the test material.

The test material was found to have an LD_{50} greater than the maximum applicable dose of 5 gm/kg. Because of the lack of significant toxic effects and because the death of animal number 9053 did not appear to be compound-related, the quantitative study (LD_{50} determination) was unnecessary. The test material was evaluated as having nondetectable (ND) toxicity based on EPA Level 1 evaluation criteria[1].

B. Tables

This report is based on the data provided in Table 2.

TABLE 2
QUANTAL TOXICITY DATA WTH WEANLING MICE

Quantal Toxicity: Weanling CD-1 mice
Sponsor: Acurex Corporation
Test Article: A81-05-030-744 (EA-2 flyash)
Description: Gray and white particles with larger (long and thin) black chunks. Sample pulverized to a fine, black powder.
Vehicle: Sterile, deionized water
Study Dates: October 8, 1981 to October 23, 1981
Animals: Charles River CD-1 mice, P.O. 106949
Dose: 5 gm/kg administered by oral gavage

Animal No.	Initial Weight gm	Final Weight gm	Visible Toxic Signs[a]	Gross Necropsy Findings
Males				
9042	10.8	19.1	NTS[b]	NSL[c]
9043	11.5	21.2	NTS	NSL
9044	11.0	20.8	NTS	NSL
9045	11.1	23.9	NTS	NSL
9046	11.6	22.4	NTS	NSL
9047	11.6	22.3	NTS	Intestines yellow and flaccid
9048	10.8	20.7	NTS	NSL
9049	11.5	21.0	NTS	NSL
9050	12.1	23.0	NTS	NSL
9051	13.1	25.8	NTS	Large white mucous plug in bladder and uretha.

Mean Body Weight: Initial = 11.5 ± 0.7 gm (Standard Deviation)
Final = 22.0 ± 1.9 gm (Standard Deviation)

---

[a] Animals observed over 14 days.
Note: Staining of the muzzle from the test material was noted in some animals after dosing. Animals seemed uncomfortable after dosing.
[b] NTS = No Toxic Signs.
[c] NSL = No Significant Lesions

TABLE 2 (Continued)
QUANTAL TOXICITY DATA WTH WEANLING MICE

Animal No.	Initial Weight gm	Final Weight gm	Visible Toxic Signs[a]	Gross Necropsy Findings
Females				
9052	11.3	18.4	NTS[b]	NSL[c]
9053	11.3	9.5	Death[d]	Animal had been dead several hours, abdomen bloated; intestines light red and filled with gas. Liver dark green colored with apparent necrosis. Lungs pale but normal. No other gross abnormalities noted.
9054	11.1	19.6	NTS	NSL
9055	13.1	20.3	NTS	NSL
9056	11.3	18.4	NTS	NSL
9057	11.6	19.4	NTS	NSL
9058	14.3	19.4	NTS	NSL
9059	11.9	19.3	NTS	NSL
9060	12.4	18.9	NTS	NSL
9061	12.0	20.3	NTS	NSL

Mean Body Weight:
Initial = 12.0 ± 1.0 gm (Standard Deviation)[e]
Final = 19.3 ± 0.7 gm (Standard Deviation)[e]

---

[a] Animals observed over 14 days.
Note: Staining of the muzzle from the test material was noted in some animals after dosing. Animals seemed uncomfortable after dosing.
[b] NTS = No Toxic Signs.
[c] NSL = No Significant Lesions
[d] Animal found dead 8:00 a.m. 10-12-81 (day 3 of the study), last seen alive 9:00 a.m. 10-11-81.
[e] Animal 9053 excluded from average.

VII. EVALUATION CRITERIA

If no mortality occurs in the quantal study, no further studies will be performed with the test substance and the LD_{50} should be reported as greater than 5 ml/kg or 5 g/kg. The test material is then ranked as having nondectable toxicity (ND) at the maximum applicable dose (MAD). Effluent samples which produce harmful effects *in vivo* and do not result in deaths will be noted in the results summary. Such observations are difficult to quantitate but provide insight into the sublethal effects of a sample on rodents. Further investigations may be recommended from observations of nonlethal toxic effects.

If a single animal in the quantal study dies in the 14-day observation period, a quantitative study will be performed. An LD_{50} will be calculated by the method of Litchfield and Wilcoxin[4]. If the data are not suitable for calculation of a precise LD_{50}, i.e., total mortality occurs for the lowest dose, an estimate of the LD_{50} could be made or the LD_{50} could be expressed as 0.05 ml/kg or 0.05 g/kg or less. Occasionally, it may be necessary to use a different series of dosages in a repeat study to accurately locate the LD_{50}. The calculated LD_{50} value is used to rank the toxicity of the test material according to the dose ranges presented in Table 3.

Frequent observations are also made and recorded on all animals through the 14-day period. As in the quantal phase, no attempt is made to quantitate or rank observations. The average animal body weight of each group is determined initially and at the termination of the experiment. The average weights and the weights as fractions of the control are reported for each dose level. Necropsy observations are recorded and reported.

TABLE 3

ACUTE *IN VIVO* RODENT TOXICITY ASSAY EVALUATION CRITERIA

Toxicity[a]	Solids (LD_{50} in g/kg)	Liquids (LD_{50} in ml/kg)
High	<0.05	<0.05
Moderate	0.05 to 0.5	0.05 to 0.5
Low	0.5 to 5	0.5 to 5
Not Detectable	>5	>5

[a] Evaluation criteria formulated by Litton Bionetics, Inc. for *IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests.*[1]

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THE ACUTE TOXICITY OF FIVE SAMPLES TO FRESHWATER ORGANISMS.

SUBMITTED TO

ACUREX CORPORATION

MOUNTAIN VIEW, CALIFORNIA

REPORT #BW-81-7-966

EG&G, Bionomics
Aquatic Toxicology Laboratory
790 Main Street
Wareham, Massachusetts
July, 1981

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INTRODUCTION

The purpose of this study was to estimate the acute toxicity of five samples received from the Acurex Corporation to freshwater organisms. All five materials were tested with the water flea (*Daphnia magna*) and the freshwater algae (*Selenastrum capricornutum*). Three of the samples were also tested with the fathead minnow (*Pimephales promelas*). Results of tests performed with water fleas and fathead minnows are reported as median lethal concentrations (LC50's) and corresponding 95% confidence intervals. Results of the tests performed with the freshwater alga are reported as the median effect concentration (EC50) and corresponding 95% confidence interval. Toxicity tests performed with water fleas and fathead minnows were conducted at the Aquatic Toxicology Laboratory of EG&G, Bionomics, Wareham, Massachusetts and the tests performed with the alga were conducted at EG&G, Bionomics Marine Research Laboratory (BMRL), Pensacola, Florida. All raw data related to these tests are stored at the respective laboratory at which they were performed.

MATERIALS AND METHODS

Methods used in performing the acute toxicity tests followed those described in "IERL-RTP Procedures Manual: Level I Environmental Assessment Biological Tests" (1980) unless specified otherwise.

The five samples were received at EG&G, Bionomics, Wareham, Massachusetts on 24 June 1981. The samples were received at ambient temperature (20-25°C) and were refrigerated (4°C) upon receipt. On 25 June, a portion of each sample was shipped to BMRL. Samples were received at BMRL on 26 June. At BMRL, the four solid samples were stored at ambient room temperature, while the liquid sample was stored at 4°C. The five samples are described in Appendix A. Tests performed with D. magna and P. promelas were limited to a high test concentration of 1000 mg/ℓ. If insufficient mortality was observed at this treatment level, the LC50 was estimated to be >1000 mg/ℓ.

Test Organisms

The water flea used in this toxicity test were obtained from laboratory stocks cultured at EG&G, Bionomics. The culture water was prepared by reconstituting deionized water (U.S. EPA, 1975) and filtering it through an Amberlite XAD-7 resin column to remove any potential organic contaminants. This water had a total hardness and akalinity as calcium carbonate ($CaCO_3$) of 170 ± 15 mg/ℓ and 120 ± 10 mg/ℓ, respectively, a pH range of 7.9-8.3, a temperature of 22 ± 1°C, a specific conductance of 400-600 micromhos per centimeter (μmhos/cm), and a dissolved oxygen (DO) concentration of greater than 5.3 mg/ℓ (60% of saturation).

The fathead minnows (Bionomics lot #81A6) were obtained from cultures spawned and raised at EG&G, Bionomics, Wareham, Massachusetts. The fish were held in a 500-ℓ fiberglass tank under a photoperiod of 16 hours light and 8 hours darkness. All fish were fed a dry, pelleted food, *ad libitum*, daily except during the 48 hours prior to testing. There was no mortality in the test fish population during this 2 day period (Daily Record of Fish Holding Conditions). The well water which flowed into this tank was characterized as having total hardness and alkalinity ranges as calcium carbonate ($CaCO_3$) of 20-25 mg/ℓ and 20-28 mg/ℓ, respectively, and a specific conductance range of 90-110 micromhos per centimeter (μmhos/cm) (Weekly Gravity Feed Tank Water Quality Analysis Logbook). Other parameters monitored in the holding tank were a pH range of 6.2-6.9, a dissolved oxygen (DO) range of 80-92% of saturation and a flow rate range of 6-7 tank volume replacements/day (Weekly Record of Fish Holding Water Characteristics). Test fish were maintained under these conditions for a minimum of 14 days. The temperature in the holding tank ranged from 20-22°C during this 14 day period (Daily Record of Fish Holding Conditions). The specific conductance was measured with a YSI Model #33 conductivity meter, the pH was measured with an Instrumentation Laboratory Model #175 pH meter and combination electrode, the DO was measured with a YSI Model #57 dissolved oxygen meter and probe and the temperature was measured with a Brooklyn alcohol thermometer. Total hardness and alkalinity were measured according to APHA et al. (1975).

The freshwater alga were obtained from the U.S. Environmental Protection Agency's Environmental Research Laboratory, Corvallis, Oregon and maintained in stock culture at BMRL. Culture procedures used for the alga followed those described in "IERL-RTP Procedures Manual: Level I Environmental Assessment Biological Tests" dated September 1980.

Test Conditions

Water flea

The toxicity tests exposing *D. magna* to the samples were conducted in 250 milliliter (mℓ) glass beakers. The dilution water used during this study was prepared in the same manner as the culture water except that the quantity of salts were reduced to yield a total hardness and alkalinity of 107 mg/ℓ as $CaCO_3$ and 69 mg/ℓ as $CaCO_3$, a pH of 8.0 and a specific conductance of 400 μmhos/cm. For each test concentration, the appropriate amount of test material was added to dilution water to total 1000 mℓ, then vigorously mixed on a magnetic stirrer. Eight hundred milliliters of this test mixture were divided into four beakers to provide replicate exposure treatments each containing 200 mℓ. The remaining 200 mℓ of the control and the high, middle and low test concentrations were used for 0-hour dissolved oxygen (DO), pH and specific conductance determinations. Four control beakers containing the same dilution water and maintained under the same conditions as the exposure concentrations, but containing no sample, were established. The ambient air temperature in the laboratory was controlled in order to maintain test solution temperatures at 21-22°C. Test solutions were not aerated. The test area was illuminated with Durotest (Optima) fluorescent lights at an intensity of 50-70 footcandles.

Twenty water flea, $\leq$24 hours old, were impartially distributed to each concentration (5 water flea per replicate) within 30 minutes after the test solutions had been prepared. Mortalities in replicate test solutions were recorded at 24 and 48 hour exposures. Biological observations and observations of the physical characteristics of each replicate test solution were also made and recorded at 0, 24 and 48 hours. The pH, DO and specific conductance were measured at 0 and 48 hours of exposure in the control and the high, middle and low test solutions. The temperature of the control and all test concentrations were measured at 0, 24, and 48 hours exposure.

Fathead minnow

Toxicity tests performed with the fathead minnow were performed in 19.6-ℓ glass jars which contained 15-ℓ of test solution. The dilution water used was hard water reconstituted from deionized water according to recommended procedures (U.S. EPA, 1975). This water had a total hardness and alkalinity as $CaCO_3$ of 94 mg/ℓ and 68 mg/ℓ, respectively, a pH of 7.9 and a specific conductance of 345 μmhos/cm (Reconstituted Water, Quality Analysis).

Test solutions were prepared by adding appropriate amounts of test material directly to test vessels containing a sufficient quantity of dilution water to total 15 ℓ. Solutions were mixed by stirring with a glass rod. Each test concentration and controls were replicated.

Two control jars containing the same dilution water as used in the exposure jars, but containing no test material, were established. All test solution temperatures were controlled by a system which maintained temperatures at 21-22°C. Test solutions were not aerated during the exposure period. The photoperiod during testing was the same as that provided during acclimation.

Ten fathead minnows with a mean (range, N=30) wet weight and total length of 0.29(0.13-0.47) grams and 33(25-38) millimeters, respectively (Fish Weights and Lengths Log) were randomly distibuted to each test jar within 10 minutes after the test solutions had been prepared.

Mortalities were recorded and removed from each test jar at 0, 24, 48, 72 and 96 hours exposure. Biological observations of the fish and observations of the physical characteristics of the test solutions were also made at each 24 hour interval. The pH and DO concentrations of the control, high, middle and low test concentrations were measured at 0, 24, 48 and 96 hours of the exposure period. Specific conductance of the control, high, middle and low test concentrations were measured at 0 hour. The temperature was measured in the control jar every 24 hours during exposure.

Freshwater algae

The toxicity tests exposing the freshwater alga to the test sample was conducted in 125 mℓ flasks each of which contained 50 mℓ of test medium. Beginning cell numbers in the test flasks were approximately 1.0 x 10^4 cells/mℓ. Triplicate cultures were employed for each of the test concentrations and control. Cultures were incubated at 24°C under approximately 2,400 lux illumination. Cell counts were made at 0- and 120-hour exposures using a hemacytometer and a Zeiss Standard 14 compound microscope. The pH of all test solutions were measured at 0 and 120 hours of exposure.

Statistical Analysis

The concentrations tested and corresponding mortality data derived from the toxicity tests exposing water flea and fathead minnows to the test materials were used to estimate median lethal concentrations (LC50) and 95% confidence intervals. The LC50 is defined as the concentration (nominal or measured) of the test compound in dilution water which caused mortality of 50% of the test animal population at the stated exposure interval. The computer program utilized (Stephan, 1978, personal communication) estimated LC50 values using one of three statistical methods in the following order of preference: moving average angle analysis, probit analysis, binomial probability. The method selected was determined by the characteristics of the data base (i.e. presence or absence of test concentrations causing 100% mortality of the test animal population, number of concentrations causing mortality of a partial number of the test animal population). The computer program scanned the data base, identified the most preferred statistical method and performed the analysis. The no discernible effect concentration was also determined for each effluent sample. The no discernible effect concentration is defined as the highest concentration tested at which there were no mortalities or observed behavioral and physical abnormalities (i.e. erratic swimming, flared carapace).

The concentrations tested and corresponding percentage decrease in cell numbers from the toxicity tests exposing the freshwater alga to the test materials were used to estimate concentrations of each sample that caused a 50% and 95% decrease in cell numbers of the exposed cultures, EC50 and EC95, respectively. Each test concentration was converted to a logarithm and the corresponding percentage decrease of cell numbers was converted to a probit (Finney, 1971). The 120-hour EC50's and EC95's and their respective 95% confidence intervals were calculated by linear regression.

RESULTS

The estimated LC50 values, 95% confidence intervals and no discernible effect concentrations for D. magna and P. promelas exposed to the test samples are presented in Table 1. Table 2 presents the estimated 120-hour EC50 and EC95 values and corresponding 95% confidence intervals for S. capricornutum exposed to the test samples. P. promelas was the least sensitive species to the effects of the test materials. None of the three samples (A80-09-023-5, A81-05-030-662, A81-05-031-765) tested with P. promelas exhibited toxicity with this organism. S. capricornutum was the most sensitive species to the toxic effects of the test materials. All of the ash samples had 120-hour EC50 values less than 400 mg/ℓ. The 48-hour LC50 values for the 5 materials tested with D. magna ranged from 680 mg/ℓ to >1000 mg/ℓ.

The nominal concentrations of the test materials and corresponding effects for the three species tested are presented in Tables 3-15. The water quality parameters measured during the toxicity tests with D. magna and P. promelas are presented in Tables 16 and 17, respectively. The pH of the test solutions measured during the tests performed with S. capricornutum are presented in Table 18.

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TABLES*

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*Results of samples other than those pertinent to this study (A81-05-030-662 and A81-05-030-744) have been purposely deleted from the original report.

Table 1. Estimated LC50 values, confidence intervals and no discernible effect concentrations for *D. magna* and *P. promelas* exposed to Acurex samples.

Sample	Species	LC50 (95% confidence interval)[a]				No discernible effect concentration (mg/ℓ)
		24 hour	48 hour	72 hour	96 hour	
A81-05-030-662	*D. magna*	>1000	680[b] (570-830)	-	-	220
	P. promelas	>1000	>1000	>1000	>1000	1000
A81-05-030-744	*D. magna*	>1000	960[b] (830-1200)	-	-	360

[a] mg/ℓ.

[b] Estimated by the moving average angle method.

Table 2. Calculated 5-day EC50's and EC95's for *Selenastrum capricornutum* exposed to the five samples provided by the Acurex Corporation. The EC values were based on decrease of cell numbers on exposed cultures as compared to the control. (The 95% confidence limits are in parentheses). Concentrations were based on milligrams of the samples per liter of algal growth medium.

Sample	EC50	EC95
A81-05-030-662	290(204-412)	853(534-1,362)
A81-05-030-744	347(328-367)	894(830-963)

Table 6. Concentrations tested, corresponding percentage mortalities and observations made during the 48-hour exposure of the water flea (Daphnia magna) to the sample coded A81-05-030-662.

Nominal concentration[a] (mg/ℓ)	24 hour					48 hour				
	A	B	C	D	$\bar{x}$	A	B	C	D	$\bar{x}$
1000	0	20	20	20	15[b]	80	60	80	80	75[b]
600	0	0	0	0	0	80	60	20	20	45[b]
360	0	0	0	0	0	20	0	0	0	5
220	0	0	0	0	0	0	0	0	0	0
130	0	0	0	0	0	0	0	0	0	0
control	0	0	0	0	0	0	0	0	0	0

[a] A dark gray colored particulate matter was present on the bottom of all mixtures of A81-05-030-662.

[b] Several daphnids were lethargic.

Table 7. Concentrations tested and corresponding percentage mortalities of fathead minnows (*Pimephales promelas*) exposed to the sample coded A81-05-030-662 for 24, 48, 72 and 96 hours.

Nominal concentration[a] (mg/ℓ)	24 hour			48 hour			72 hour			96 hour		
	A	B	$\bar{x}$	A	B	$\bar{x}$	A	B	$\bar{x}$	A	B	$\bar{x}$
1000[b]	0	0	0	0	0	0	0	0	0	0	0	0
600[b]	0	0	0	0	0	0	0	0	0	0	0	0
360	0	0	0	0	0	0	0	0	0	0	0	0
220	0	0	0	0	0	0	0	0	0	0	0	0
130	0	0	0	0	0	0	0	0	0	0	0	0

[a] A dark gray particulate matter was present in all mixtures of A81-05-030-662.

[b] Mixtures were cloudy at 0 hour of exposure.

Table 8. Results of a 5-day exposure of the freshwater algae *Selenastrum capricornutum* to A81-05-030-662. Percentage change is decrease of cell numbers in exposed cultures as compared to the control at day 5.

Nominal concentration (mg/ℓ)	Percentage change
control	-
125	-7
250	-56
500	-84
1,000	-94

Table 9. Concentrations tested, corresponding percentage mortalities and observations made during the 48-hour exposure of the water flea (Daphnia magna) to the sample coded A81-05-030-744.

Nominal concentration[a] (mg/ℓ)	24 hour					48 hour				
	A	B	C	D	$\bar{x}$	A	B	C	D	$\bar{x}$
1000	0	0	0	0	0[b]	20	40	80	80	55[b,c]
600	0	0	0	0	0[b]	0	20	0	0	5[b]
360	0	0	0	0	0	0	0	0	0	0
220	0	0	0	0	0	0	0	0	0	0
130	0	0	0	0	0	0	0	0	0	0
control	0	0	0	0	0	0	0	0	0	0

[a] A gray-black colored particulate matter was present in all test mixtures of A81-05-030-744.

[b] Particulate matter was adhering to many daphnids.

[c] Several daphnids were lethargic.

Table 10. Results of a 5-day exposure of the freshwater alga *Selenastrum capricornutum* to A81-05-030-744. Percentage change is decrease of cell numbers in exposed cultures as compared to the control at day 5.

Nominal concentration (mg/ℓ)	Percentage change
control	-
125	-4
250	-29
500	-71
1,000	-97

Table 16. Water quality characterization of the test solutions measured during the acute toxicity tests exposing the water flea (*Daphnia magna*) to the Acurex test samples.

Sample	Nominal concentration (mg/ℓ)	Dissolved[a] oxygen (mg/ℓ)	pH[a]	Specific[a] conductance (μmhos/cm)
A81-05-030-662	1000	8.1-8.2	10.1-9.2	430-420
	360	8.1-8.3	9.2-8.8	370-400
	130	8.0-7.9	8.9-8.5	360-370
	control	7.5-7.6	8.1-8.1	350-360
A81-05-030-744	1000	8.2-8.1	10.2-9.0	380-380
	360	8.2-8.0	9.5-8.7	370-380
	130	8.3-8.0	8.9-8.3	350-360
	control	7.5-7.6	8.1-8.1	350-360

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[a] 0-48 hour measurements.

Table 17. Water quality parameters measured during 96-hour toxicity tests with Acurex test samples and fathead minnow (*Pimephales promelas*).

Sample	Parameter	Nominal concentration (mg/ℓ)	0 hour	24 hour	48 hour	72 hour	96 hour
A81-05-030-662	pH	1000	9.9	10.0	9.8	9.6	9.4
		360	9.4	9.2	9.1	8.9	8.8
		130	8.5	8.6	8.4	8.3	8.2
		control	8.0	7.5	7.3	7.3	7.3
	DO (mg/ℓ)	1000	8.7	6.9	5.8	5.8	5.6
		360	8.6	7.6	6.8	6.8	6.2
		130	8.6	7.5	6.1	6.7	6.6
		control	8.5	7.7	7.4	5.5	4.6
	specific conductance (μmhos/cm)	1000	380	-	-	-	-
		600	370	-	-	-	-
		360	360	-	-	-	-
		220	350	-	-	-	-
		130	345	-	-	-	-
		control	340	-	-	-	-

Table 18. pH's of test solutions during the acute toxicity tests exposing the freshwater alga (Selenastrum capricornutum) to Acurex test samples.

Sample	Nominal concentration (ppm)	pH 0 hour	pH 120 hour
A81-05-030-662	1000	10.5	8.9
	500	10.2	8.6
	250	9.9	9.7
	125	9.4	8.9
	control	7.2	8.2
A81-05-030-744	1000	10.4	8.8
	500	10.1	8.6
	250	9.5	9.2
	125	9.3	8.5
	control	7.2	8.2

Sample code	Sample name	Species tested	Amount of sample received	Sample description
A81-05-030-662	EA-1 flyash	*D. magna* *P. promelas* *S. capricornutum*	100 g	dark gray ash with white flakes
A81-05-030-744	EA-2 flyash	*D. magna* *S. capricornutum*	20 g	dark gray ash

_D_. _magna_ and _P_. _promelas_ tests

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